PCT CA 00/00227

MARCH 2000 (27.03.00)

RECD 11 APR 2000

WIPO

PA 220675

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

4

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

March 14, 2000

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: *09/262,927*
FILING DATE: *March 05, 1999*

PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

N. WILLIAMS
Certifying Officer

PATENT APPLICATION TRANSMITTAL LETTER (Large Entity)	Docket No. 1038-922 MIS:jb

TO THE ASSISTANT COMMISSIONER FOR PATENTS

JC598 U.S. PTO 03/05/99

JC618 U.S. PTO 09/262927 03/05/99

Transmitted herewith for filing under 35 U.S.C. 111 and 37 C.F.R. 1.53 is the patent application of:

Yvonne Li, et al.

For: NUCLEIC ACID RESPIRATORY SYNCYTIAL VIRUS VACCINES

Enclosed are:

- ☐ Certificate of Mailing with Express Mail Mailing Label No.
- ☒ **Thirty-four (34)** sheets of drawings.
- ☒ A ~~certified~~ copy of a specification ~~application~~
- ☒ Declaration ☐ Signed. ☒ Unsigned.
- ☐ Power of Attorney
- ☐ Information Disclosure Statement
- ☐ Preliminary Amendment
- ☐ Other:

CLAIMS AS FILED

For	#Filed	#Allowed	#Extra		Rate	Fee
Total Claims	54	- 20 =	34	x	$18.00	$612.00
Indep. Claims	11	- 3 =	8	x	$78.00	$624.00
Multiple Dependent Claims (check if applicable) ☐						$0.00
					BASIC FEE	$760.00
					TOTAL FILING FEE	$1,996.00

☒ A check in the amount of **$1,996.00** to cover the filing fee is enclosed.

☒ The Commissioner is hereby authorized to charge and credit Deposit Account No. **19-2253** as described below. A duplicate copy of this sheet is enclosed.

- ☐ Charge the amount of as filing fee.
- ☒ Credit any overpayment.
- ☒ Charge any additional filing fees required under 37 C.F.R. 1.16 and 1.17.
- ☐ Charge the issue fee set in 37 C.F.R. 1.18 at the mailing of the Notice of Allowance, pursuant to 37 C.F.R. 1.311(b).

Dated: **March 4, 1999**

Michael I. Stewart *Signature* (24,973)

cc:

P01LARGE/REV06

TITLE OF INVENTION

NUCLEIC ACID RESPIRATORY SYNCYTIAL VIRUS VACCINES

REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of copending United States Application No. 08/896,500 filed July 18, 1997, which itself is a continuation-in-part of copending United States Patent Application No. 08/659,939 filed June 7, 1996 (now U.S. Patent No. 5,843,913), which itself is continuation-in-part of copending United States Patent Application No. 08/476,397, filed June 7, 1995.

FIELD OF INVENTION

The present invention is related to the field of Respiratory Syncytial Virus (RSV) vaccines and is particularly concerned with vaccines comprising nucleic acid sequences encoding the fusion (F) protein of RSV.

BACKGROUND OF INVENTION

Respiratory syncytial virus (RSV), a negative-strand RNA virus belonging to the *Paramyxoviridae* family of viruses, is the major viral pathogen responsible for bronchiolitis and pneumonia in infants and young children (ref. 1 - Throughout this application, various references are referred to in parenthesis to more fully describe the state of the art to which this invention pertains. Full bibliographic information for each citation is found at the end of the specification, immediately preceding the claims. The disclosures of these references are hereby incorporated by reference into the present disclosure). Acute respiratory tract infections caused by RSV result in approximately 90,000 hospitalizations and 4,500 deaths per year in the United States (ref. 2). Medical care costs due to RSV infection are greater than $340 M annually in the United States alone (ref. 3). There is currently no licensed vaccine against RSV. The main approaches for developing

an RSV vaccine have included inactivated virus, live-attenuated viruses and subunit vaccines.

The F protein of RSV is considered to be one of the most important protective antigens of the virus. There is a significant similarity (89% identity) in the amino acid sequences of the F proteins from RSV subgroups A and B (ref. 3) and anti-F antibodies can cross-neutralize viruses of both subgroups as well as protect immunized animals against infection with viruses from both subgroups (ref. 4). Furthermore, the F protein has been identified as a major target for RSV-specific cytotoxic T-lymphocytes in mice and humans (ref. 3 and ref. 5).

The use of RSV proteins as vaccines may have obstacles. Parenterally administered vaccine candidates have so far proven to be poorly immunogenic with regard to the induction of neutralizing antibodies in seronegative humans or chimpanzees. The serum antibody response induced by these antigens may be further diminished in the presence of passively acquired antibodies, such as the transplacentally acquired maternal antibodies which most young infants possess. A subunit vaccine candidate for RSV consisting of purified fusion glycoprotein from RSV infected cell cultures and purified by immunoaffinity or ion-exchange chromatography has been described (ref. 6). Parenteral immunization of seronegative or seropositive chimpanzees with this preparation was performed and three doses of 50 µg were required in seronegative animals to induce an RSV serum neutralizing titre of approximately 1:50. Upon subsequent challenge of these animals with wild-type RSV, no effect of immunization on virus shedding or clinical disease could be detected in the upper respiratory tract. The effect of immunization with this vaccine on virus shedding in the lower respiratory tract was not investigated, although this is the site where

the serum antibody induced by parenteral immunization may be expected to have its greatest effect. Safety and immunogenicity studies have been performed in a small number of seropositive individuals. The vaccine was found to be safe in seropositive children and in three seronegative children (all > 2.4 years of age). The effects of immunization on lower respiratory tract disease could not be determined because of the small number of children immunized. One immunizing dose in seropositive children induced a 4-fold increase in virus neutralizing antibody titres in 40 to 60% of the vaccinees. Thus, insufficient information is available from these small studies to evaluate the efficacy of this vaccine against RSV-induced disease. A further problem facing subunit RSV vaccines is the possibility that inoculation of seronegative subjects with immunogenic preparations might result in disease enhancement (sometimes referred to as immunopotentiation), similar to that seen in formalin inactivated RSV vaccines. In some studies, the immune response to immunization with RSV F protein or a synthetic RSV FG fusion protein resulted in a disease enhancement in rodents resembling that induced by a formalin-inactivated RSV vaccine. The association of immunization with disease enhancement using non-replicating antigens suggests caution in their use as vaccines in seronegative humans.

Live attenuated vaccines against disease caused by RSV may be promising for two main reasons. Firstly, infection by a live vaccine virus induces a balanced immune response comprising mucosal and serum antibodies and cytotoxic T-lymphocytes. Secondly, infection of infants with live attenuated vaccine candidates or naturally acquired wild-type virus is not associated with enhanced disease upon subsequent natural reinfection. It will be challenging to produce live

attenuated vaccines that are immunogenic for younger infants who possess maternal virus-neutralizing antibodies and yet are attenuated for seronegative infants greater than or equal to 6 months of age. Attenuated live virus vaccines also have the risks of residual virulence and genetic instability.

Injection of plasmid DNA containing sequences encoding a foreign protein has been shown to result in expression of the foreign protein and the induction of antibody and cytotoxic T-lymphocyte responses to the antigen in a number of studies (see, for example, refs. 7, 8, 9). The use of plasmid DNA inoculation to express viral proteins for the purpose of immunization may offer several advantages over the strategies summarized above. Firstly, DNA encoding a viral antigen can be introduced in the presence of antibody to the virus itself, without loss of potency due to neutralization of virus by the antibodies. Secondly, the antigen expressed *in vivo* should exhibit a native conformation and, therefore, should induce an antibody response similar to that induced by the antigen present in the wild-type virus infection. In contrast, some processes used in purification of proteins can induce conformational changes which may result in the loss of immunogenicity of protective epitopes and possibly immunopotentiation. Thirdly, the expression of proteins from injected plasmid DNAs can be detected *in vivo* for a considerably longer period of time than that in virus-infected cells, and this has the theoretical advantage of prolonged cytotoxic T-cell induction and enhanced antibody responses. Fourthly, *in vivo* expression of antigen may provide protection without the need for an extrinsic adjuvant.

The ability to immunize against disease caused by RSV by administration of a DNA molecule encoding an RSV F protein was unknown before the present invention. In

particular, the efficacy of immunization against RSV induced disease using a gene encoding a secreted form of the RSV F protein was unknown. Infection with RSV leads to serious disease. It would be useful and desirable to provide isolated genes encoding RSV F protein and vectors for *in vivo* administration for use in immunogenic preparations, including vaccines, for protection against disease caused by RSV and for the generation of diagnostic reagents and kits. In particular, it would be desirable to provide vaccines that are immunogenic and protective in humans, including seronegative infants, that do not cause disease enhancement (immunopotentiation).

SUMMARY OF INVENTION

The present invention relates to a method of immunizing a host against disease caused by respiratory syncytial virus, to nucleic acid molecules used therein, and to diagnostic procedures utilizing the nucleic acid molecules. In particular, the present invention is directed towards the provision of nucleic acid respiratory syncytial virus vaccines.

In accordance with one aspect of the invention, there is provided an immunogenic composition for *in vivo* administration to a host for the generation in the host of a protective immune response to RSV F protein, comprising a non-replicating vector comprising:

a first nucleotide sequence encoding an RSV F protein or a RSV F protein fragment that generates antibodies and/or cytotoxic T-lymphocytes (CTLs) that specifically react with RSV F protein;

a promoter sequence operatively coupled to the first nucleotide sequence for expression of the RSV F protein, and

a second nucleotide sequence located adjacent the first nucleotide sequence to enhance the

immunoprotective ability of the RSV F protein when expressed *in vivo* from the vector in a host; and

a pharmaceutically-acceptable carrier therefor.

The first nucleotide sequence may be that which encodes a full-length RSV F protein, as seen in Figure 2 (SEQ ID No: 2). Alternatively, the first nucleotide sequence may be that which encodes an RSV F protein from which the transmembrane region is absent. The latter embodiment may be provided by a nucleotide sequence which encodes a full-length RSV F protein but contains a translational stop codon immediately upstream of the start of the transmembrane coding region, thereby preventing expression of a transmembrane region of the RSV F protein, as seen in Figure 3 (SEQ. ID No. 4). The lack of expression of the transmembrane region results in a secreted form of the RSV F protein.

The first nucleotide sequence may encode a RSV F protein fragment lacking an autologous RSV F signal peptide sequence and may include, in its place, a sequence encoding a heterologous signal peptide sequence which enhances the level of expression of the RSV F protein. One signal peptide which has been found useful in this regard is the signal peptide of Herpes Simplex Virus I (HSV I)gD. Such enhanced expression levels also lead to improve immunogenicity of the vector at the same dosage level. The first nucleotide sequence may also encode a RSV F protein fragment lacking a transmembrane coding region.

The second nucleotide sequence may comprise a pair of splice sites to prevent aberrant mRNA splicing, whereby substantially all transcribed mRNA encodes the RSV protein. Such second nucleotide sequence may be located between the first nucleotide sequence and the promoter sequence. Such second nucleotide sequence may be that of rabbit β-globin intron II, as shown in Figure 8 (SEQ ID No: 5).

A vector encoding the F protein and provided by this aspect of the invention may specifically be pXL2 or pXL4 or p82M35B, as seen in Figures 5, 7 or 10, respectively.

The promoter sequence may be an immediate early cytomegalovirus (CMV) promoter.

Certain of the vectors provided herein may be used to immunize a host against RSV infection or disease by *in vivo* expression of RSV F protein lacking a transmembrane region following administration of the vectors. In accordance with a further aspect of the present invention, therefore, there is provided a method of immunizing a host against disease caused by infection with respiratory syncytial virus, which comprises administering to the host an effective amount a of non-replicating vector comprising a first nucleotide sequence encoding an RSV F protein or a RSV F protein fragment that generates antibodies and/or CTLs that specifically react with RSV F protein and a promoter sequence operatively coupled to the first nucleotide sequence for expression of the RSV F protein in the host, which may be a human. The promoter may be an immediate early cytomegalovirus promoter.

The nucleotide sequence may encode a truncated RSV F protein lacking the transmembrane region may be that as described above and/or possess a heterologous signal peptide encoding sequence.

The vector may contain a second nucleotide sequence located adjacent a first nucleotide sequence and effective to enhance the immunoprotective ability of the RSV F protein expressed by the first nucleotide sequence may be used to immunize a host. Specific non-replicating vectors which may be used in this aspect of the invention are those identified as plasmid vectors pXL2, pXL4 and p82M35B in Figures 5, 7 and 10 respectively.

The present invention also includes a novel method of using a gene encoding an RSV F protein or a RSV F protein fragment that generates antibodies and/or CTLs that specifically react with RSV F protein to protect a host against disease caused by infection with respiratory syncytial virus, which comprises:

isolating the gene;

operatively linking the gene to at least one control sequence to produce a non-replicating vector, said control sequence directing expression of the RSV F protein when said vector is introduced into a host to produce an immune response to the RSV F protein or fragment thereof, and

introducing the vector into the host.

The procedure provided in accordance with this aspect of the invention may further include the step of:

operatively linking the gene to an immunoprotection enhancing sequence to produce an enhanced immunoprotection by the RSV F protein in the host, preferably by introducing the immunoprotection enhancing sequence between the control sequence and the gene.

In addition, the present invention includes a method of producing a vaccine for protection of a host against disease caused by infection with respiratory syncytial virus, which comprises:

isolating a first nucleotide sequence encoding an RSV F protein or a RSV F protein fragment that generates antibodies and/or CLTs that specifically react with RSV F protein;

operatively linking the first nucleotide sequence to at least one control sequence to produce a non-replicating vector, the control sequence directing expression of the RSV F protein when introduced into a host to produce an immune response to the RSV F protein when expressed *in vivo* from the vector in a host, and

formulating the vector as a vaccine for *in vivo* administration.

The first nuclectide sequence further may be operatively linked to a second nucleotide sequence to enhance the immunoprotective ability of the RSV F protein when expressed *in vivo* from the vector in a host. The vector may be a plasmid vector selected from pXL2, pXL4 and p82M35B. The invention further includes a vaccine for administration to a host, including a human host, produced by this method as well as immunogenic compositions comprising an immunoeffective amount of the vectors described herein.

As noted previously, the vectors provided herein are useful in diagnostic applications. In a further aspect of the invention, therefore, there is provided a method of determining the presence of an RSV F protein in a sample, comprising the steps of:

(a) immunizing a host with a non-replicating vector comprising a first nucleotide sequence encoding an RSV F protein or a RSV F protein fragment that generates antibodies and/or cytotoxic T-lymphocytes (CTLs) that specifically react with RSV F protein and a promoter sequence operatively coupled to the first nucleotide sequence for expression of the RSV F protein in the host to produce antibodies specific for the RSV F protein;

(b) isolating the RSV F protein specific antibodies;

(c) contacting the sample with the isolated antibodies to produce complexes comprising any RSV F protein present in the sample and the RSV F protein- specific antibodies; and

(d) determining production of the complexes.

The non-replicating vector employed to elicit the antibodies may be a plasmid vector which is pXL1, pXL2, pXL3, pXL4 or p82M35B.

The invention also includes a diagnostic kit for detecting the presence of an RSV F protein in a sample, comprising:

(a) a non-replicating vector comprising a first nucleotide sequence encoding an RSV F protein or a RSV F protein fragment that generates antibodies that specifically react with RSV F protein and a promoter sequence operatively coupled to said first nucleotide sequence for expression of said RSV F protein in a host immunized therewith to produce antibodies specific for the RSV F protein;

(b) isolation means to isolate said RSV F protein specific antibodies;

(c) contacting means to contact the isolated RSV F specific antibodies with the sample to produce a complex comprising any RSV F protein present in the sample and RSV F protein specific antibodies; and

(d) identifying means to determine production of the complex.

The present invention is further directed to a method for producing RSV F protein specific polyclonal antibodies comprising the use of the immunization method described herein, and further comprising the step of isolating the RSV F protein specific polyclonal antibodies from the immunized animal.

The present invention is also directed to a method for producing monoclonal antibodies specific for an F protein of RSV, comprising the steps of:

(a) constructing a non-replicating vector comprising a first nucleotide sequence encoding a RSV F protein and a promoter sequence operatively coupled to said first nucleotide sequence for expression of said RSV F protein; and, optionally,

a second nucleotide sequence located adjacent said first nucleotide sequence to enhance the

immunoprotective ability of said RSV F protein when expressed *in vivo* from said vector in a host.

(b) administering the vector to at least one mouse to produce at least one immunized mouse;

(c) removing B-lymphocytes from the at least one immunized mouse;

(d) fusing the B-lymphocytes from the at least one immunized mouse with myeloma cells, thereby producing hybridomas;

(e) cloning the hybridomas;

(f) selecting clones which produce anti-F protein antibody;

(g) culturing the anti-F protein antibody-producing clones; and

(h) isolating anti-F protein monoclonal antibodies.

In this application, the term "RSV F protein" is used to define (1) a full-length RSV F protein, such proteins having variations in their amino acid sequences including those naturally occurring in various strains of RSV, (2) a secreted form of RSV F protein lacking a transmembrane region, and (3) functional analogs of the RSV F protein. In this application, a first protein is a "functional analog" of a second protein if the first protein is immunologically related to and/or has the same function as the second protein. The functional analog may be, for example, a fragment of the protein or a substitution, addition or deletion mutant thereof. Included are RSV F protein fragments that generate antibodies and/or CTLs that specifically react with RSV F protein.

BRIEF DESCRIPTION OF THE FIGURES

The present invention will be further understood from the following General Description and Examples with reference to the Figures in which:

Figure 1 illustrates a restriction map of the gene encoding the F protein of Respiratory Syncytial Virus;

Figures 2A, 2B, 2C, 2D and 2E show the nucleotide sequence of the gene encoding the membrane attached form of the F protein of Respiratory Syncytial Virus (SEQ ID No: 1) as well as the amino acid sequence of the RSV F protein encoded thereby (SEQ ID No: 2);

Figures 3A, 3B, 3C and 3D show the nucleotide sequence of the gene encoding the secreted form of the RSV F protein lacking the transmembrane region (SEQ ID No: 3) as well as the amino acid sequence of the truncated RSV F protein lacking the transmembrane region encoded thereby (SEQ ID No: 4);

Figures 4A, 4B, 4C and 4D show the construction of plasmid pXL1 containing the gene encoding a secreted form of the RSV F protein lacking the transmembrane region;

Figures 5A, 5B, 5C and 5D show the construction of plasmid pXL2 containing a gene encoding a secreted form of the RSV F protein lacking the transmembrane region and containing the rabbit β-globin Intron II sequence;

Figures 6A, 6B, 6C and 6D show the construction of plasmid pXL3 containing the gene encoding a full length membrane attached form of the RSV F protein;

Figure 7 shows the construction of plasmid pXL4 containing a gene encoding a membrane attached form of the RSV F protein and containing the rabbit β-globin Intron II sequence;

Figure 8 shows the nucleotide sequence for the rabbit β-globin Intron II sequence (SEQ ID No. 5);

Figure 9 shows the lung cytokine expression profile in DNA-immunized mice after RSV challenge;

Figure 10 is a schmmatic showing the assembly of plasmid p82M35B containing a gene encoding a secreted form of the RSV F protein lacking the transmembrane

region, the rabbit β-globin Intron II sequence and the signal peptide sequence HSV I gD;

Figure 11 shows the nucleotide sequence of plasmid VR-1012 (SEQ ID No: 6); and

Figure 12 shows DNA (SEQ ID No: 7) and derived amino acid (SEQ ID No: 8) sequence of the HSV gD signal peptide sequence, synthesized as a synthetic oligopeptide.

GENERAL DESCRIPTION OF INVENTION

As described above, the present invention relates generally to polynucleotide, including DNA, immunization to obtain protection against infection by respiratory syncytial virus (RSV) and to diagnostic procedures using particular vectors. In the present invention, several recombinant vectors were constructed to contain a nucleotide sequence encoding an RSV F protein.

The nucleotide sequence of the full length RSV F gene is shown in Figure 2 (SEQ ID No: 1). Certain constructs provided herein include the nucleotide sequence encoding the full-length RSV F (SEQ ID No: 2) protein while others include an RSV F gene modified by insertion of termination codons immediately upstream of the transmembrane coding region (see Figure 3, SEQ ID No: 3), to prevent expression of the transmembrane portion of the protein and to produce a secreted or truncated RSV F protein lacking a transmembrane region (SEQ ID No. 4). In addition, certain constructs provided herein include a nucleic acid sequence encoding a heterologous signal peptide sequence rather than the native signal peptide sequence to provide for enhanced protein expression and increased immunogenicity. Specifically, the signal peptide sequence for HSV I gD is employed. However, other heterologous signal peptides may be employed, such as that of human tissue plasminogen activator (TPA).

The nucleotide sequence encoding the RSV F protein is operatively coupled to a promoter sequence for expression of the encoded RSV F protein. The promoter sequence may be the immediately early cytomegalovirus (CMV) promoter. This promoter is described in ref. 13. Any other convenient promoter may be used, including constitutive promoters, such as, Rous Sarcoma Virus LTRs, and inducible promoters, such as metallothionine promoter, and tissue specific promoters.

The vectors provided herein, when administered to an animal, effect *in vivo* RSV F protein expression, as demonstrated by an antibody response in the animal to which it is administered. Such antibodies may be used herein in the detection of RSV protein in a sample, as described in more detail below. When the encoded RSV F protein is in the form of an RSV F protein from which the transmembrane region is absent, such as plasmid pXL1 (Figure 4), the administration of the vector conferred protection in mice and cotton rats to challenge by live RSV virus neutralizing antibody and cell mediated immune responses and an absence of immunopotentiation in immunized animals, as seen from the Examples below.

The recombinant vector also may include a second nucleotide sequence located adjacent the RSV F protein encoding nucleotide sequence to enhance the immunoprotective ability of the RSV F protein when expressed *in vivo* in a host. Such enhancement may be provided by increased *in vivo* expression, for example, by increased mRNA stability, enhanced transcription and/or translation. This additional sequence preferably is located between the promoter sequence and the RSV F protein-encoding sequence.

This enhancement sequence may comprise a pair of splice sites to prevent aberrant mRNA splicing during transcription and translation so that substantially all transcribed mRNA encodes an RSV F protein.

Specifically, the rabbit β-globin Intron II sequence shown in Figure 7 (SEQ ID No: 5) may provide such splice sites, as also described in ref. 15.

The construct containing the Intron II sequence, CMV promoter and nucleotide sequence coding for the truncated RSV F protein lacking a transmembrane region, i.e. plasmid pXL2 (Figure 5), induced complete protection in mice against challenge with live RSV, as seen in the Examples below. In addition, the construct containing the Intron II sequence, CMV promoter and nucleotide sequence coding for the full-length RSV F protein, i.e. plasmid pXL4 (Figure 7), also conferred protection in mice to challenge with live RSV, as seen from the Examples below. The construct containing the Intron II sequence, CMV promoter, HSV I gD signal peptide peptide encoding sequence and nucleotide sequence coding for the truncated RSV F protein lacking a transmembrane region, i.e. plasmid p82M35B (Figure 10), induced complete protection in the absence of cardotoxin pretreatment under conditions where pretreatment with cardiotoxin was required for pXL2 to confer complete protection, as seen from the Examples below.

The vector provided herein may also comprise a third nucleotide sequence encoding a further antigen from RSV, an antigen from at least one other pathogen or at least one immunomodulating agent, such as cytokine. Such vector may contain said third nucleotide sequence in a chimeric or a bicistronic structure. Alternatively, vectors containing the third nucleotide sequence may be separately constructed and coadministered to a host, with the nucleic acid molecule provided herein.

It is clearly apparent to one skilled in the art, that the various embodiments of the present invention have many applications in the fields of vaccination, diagnosis and treatment of RSV infections. A further

non-limiting discussion of such uses is further presented below.

1. Vaccine Preparation and Use

Immunogenic compositions, suitable to be used as vaccines, may be prepared from the RSV F genes and vectors as disclosed herein. The vaccine elicits an immune response in a subject which includes the production of anti-F antibodies. Immunogenic compositions, including vaccines, containing the nucleic acid may be prepared as injectables, in physiologically-acceptable liquid solutions or emulsions for polynucleotide administration. The nucleic acid may be associated with liposomes, such as lecithin liposomes or other liposomes known in the art, as a nucleic acid liposome (for example, as described in WO 9324640, ref. 17) or the nucleic acid may be associated with an adjuvant, as described in more detail below. Liposomes comprising cationic lipids interact spontaneously and rapidly with polyanions such as DNA and RNA, resulting in liposome/nucleic acid complexes that capture up to 100% of the polynucleotide. In addition, the polycationic complexes fuse with cell membranes, resulting in an intracellular delivery of polynucleotide that bypasses the degradative enzymes of the lysosomal compartment. Published PCT application WO 94/27435 describes compositions for genetic immunization comprising cationic lipids and polynucleotides. Agents which assist in the cellular uptake of nucleic acid, such as calcium ions, viral proteins and other transfection facilitating agents, may advantageously be used.

Polynucleotide immunogenic preparations may also be formulated as microcapsules, including biodegradable time-release particles. Thus, U.S. Patent 5,151,264 describes a particulate carrier of a phospholipid/glycolipid/polysaccharide nature that has

been termed Bio Vecteurs Supra Moléculaires (BVSM). The particulate carriers are intended to transport a variety of molecules having biological activity in one of the layers thereof.

U.S. Patent 5,075,109 describes encapsulation of the antigens trinitrophenylated keyhole limpet hemocyanin and staphylococcal enterotoxin B in 50:50 poly (DL-lactideco-glycolide). Other polymers for encapsulation are suggested, such as poly(glycolide), poly(DL-lactide-co-glycolide), copolyoxalates, polycaprolactone, poly(lactide-co-caprolactone), poly(esteramides), polyorthoesters and poly(8-hydroxybutyric acid), and polyanhydrides.

Published PCT application WO 91/06282 describes a delivery vehicle comprising a plurality of bioadhesive microspheres and antigens. The microspheres being of starch, gelatin, dextran, collagen or albumin. This delivery vehicle is particularly intended for the uptake of vaccine across the nasal mucosa. The delivery vehicle may additionally contain an absorption enhancer.

The RSV F genes and vectors may be mixed with pharmaceutically acceptable excipients which are compatible therewith. Such excipients may include, water, saline, dextrose, glycerol, ethanol, and combinations thereof. The immunogenic compositions and vaccines may further contain auxiliary substances, such as wetting or emulsifying agents, pH buffering agents, or adjuvants to enhance the effectiveness thereof. Immunogenic compositions and vaccines may be administered parenterally, by injection subcutaneously, intravenously, intradermally or intramuscularly, possibly following pretreatment of the injection site with a local anesthetic. Alternatively, the immunogenic compositions formed according to the present invention, may be formulated and delivered in a manner to evoke an immune response at mucosal surfaces. Thus, the

immunogenic composition may be administered to mucosal surfaces by, for example, the nasal or oral (intragastric) routes. Alternatively, other modes of administration including suppositories and oral formulations may be desirable. For suppositories, binders and carriers may include, for example, polyalkalene glycols or triglycerides. Oral formulations may include normally employed incipients, such as, for example, pharmaceutical grades of saccharine, cellulose and magnesium carbonate.

The immunogenic preparations and vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective, protective and immunogenic. The quantity to be administered depends on the subject to be treated, including, for example, the capacity of the individual's immune system to synthesize the RSV F protein and antibodies thereto, and if needed, to produce a cell-mediated immune response. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner. However, suitable dosage ranges are readily determinable by one skilled in the art and may be of the order of about 1 μg to about 1 mg of the RSV F genes and vectors. Suitable regimes for initial administration and booster doses are also variable, but may include an initial administration followed by subsequent administrations. The dosage may also depend on the route of administration and will vary according to the size of the host. A vaccine which protects against only one pathogen is a monovalent vaccine. Vaccines which contain antigenic material of several pathogens are combined vaccines and also belong to the present invention. Such combined vaccines contain, for example, material from various pathogens or from various strains of the same pathogen, or from combinations of various pathogens.

Immunogenicity can be significantly improved if the vectors are co-administered with adjuvants, commonly used as 0.05 to 0.1 percent solution in phosphate-buffered saline. Adjuvants enhance the immunogenicity of an antigen but are not necessarily immunogenic themselves. Adjuvants may act by retaining the antigen locally near the site of administration to produce a depot effect facilitating a slow, sustained release of antigen to cells of the immune system. Adjuvants can also attract cells of the immune system to an antigen depot and stimulate such cells to elicit immune responses.

Immunostimulatory agents or adjuvants have been used for many years to improve the host immune responses to, for example, vaccines. Thus, adjuvants have been identified that enhance the immune response to antigens. Some of these adjuvants are toxic, however, and can cause undesirable side-effects, making them unsuitable for use in humans and many animals. Indeed, only aluminum hydroxide and aluminum phosphate (collectively commonly referred to as alum) are routinely used as adjuvants in human and veterinary vaccines.

A wide range of extrinsic adjuvants and other immunomodulating material can provoke potent immune responses to antigens. These include saponins complexed to membrane protein antigens to produce immune stimulating complexes (ISCOMS), pluronic polymers with mineral oil, killed mycobacteria in mineral oil, Freund's complete adjuvant, bacterial products, such as muramyl dipeptide (MDP) and lipopolysaccharide (LPS), as well as monophoryl lipid A, QS 21 and polyphosphazene.

In particular embodiments of the present invention, the vector comprising a first nucleotide sequence encoding an F protein of RSV may be delivered in conjunction with a targeting molecule to target the

vector to selected cells including cells of the immune system.

The polynucleotide may be delivered to the host by a variety of procedures, for example, Tang et al. (ref. 10) disclosed that introduction of gold microprojectiles coated with DNA encoding bovine growth hormone (BGH) into the skin of mice resulted in production of anti-BGH antibodies in the mice, while Furth et al. (ref. 11) showed that a jet injector could be used to transfect skin, muscle, fat and mammary tissues of living animals.

2. Immunoassays

The RSV F genes and vectors of the present invention are useful as immunogens for the generation of anti-F antibodies for use in immunoassays, including enzyme-linked immunosorbent assays (ELISA), RIAs and other non-enzyme linked antibody binding assays or procedures known in the art. In ELISA assays, the vector first is administered to a host to generate antibodies specific to the RSV F protein. These RSV F-specific antibodies are immobilized onto a selected surface, for example, a surface capable of binding the antibodies, such as the wells of a polystyrene microtiter plate. After washing to remove incompletely adsorbed antibodies, a nonspecific protein such as a solution of bovine serum albumin (BSA) that is known to be antigenically neutral with regard to the test sample may be bound to the selected surface. This allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus reduces the background caused by nonspecific bindings of antisera onto the surface.

The immobilizing surface is then contacted with a sample, such as clinical or biological materials, to be tested in a manner conducive to immune complex (antigen/antibody) formation. This procedure may include diluting the sample with diluents, such as

solutions of BSA, bovine gamma globulin (BGG) and/or phosphate buffered saline (PBS)/Tween. The sample is then allowed to incubate for from about 2 to 4 hours, at temperatures such as of the order of about 20° to 37°C. Following incubation, the sample-contacted surface is washed to remove non-immunocomplexed material. The washing procedure may include washing with a solution, such as PBS/Tween or a borate buffer. Following formation of specific immunocomplexes between the test sample and the bound RSV F specific antibodies, and subsequent washing, the occurrence, and even amount, of immunocomplex formation may be determined.

BIOLOGICAL MATERIALS

Certain plasmids that contain the gene encoding RSV F protein and referred to herein have been deposited with the America Type Culture Collection (ATCC) located at 10801 University Blvd., Manassas, VA 20110-2209, U.S.A., pursuant to the Budapest Treaty and prior to the filing of this application.

Samples of the deposited plasmids will become available to the public upon grant of a patent based upon this United States patent application and all restrictions on access to the deposits will be removed at that time. The deposits will be replaced if the Depository is unable to dispense viable samples. The invention described and claimed herein is not to be limited in scope by plasmids deposited, since the deposited embodiment is intended only as an illustration of the invention. Any equivalent or similar plasmids that encode similar or equivalent antigens as described in this application are within the scope of the invention.

Plasmid	ATCC Designation	Date Deposited
pXL1	97167	May 30, 1995
pXL2	97168	May 30, 1995
pXL3	97169	May 30, 1995
pXL4	97170	May 30, 1995
p82M35B		

EXAMPLES

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific Examples. These Examples are described solely for purposes of illustration and are not intended to limit the scope of the invention. Changes in form and substitution of equivalents are contemplated as circumstances may suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitations.

Methods of molecular genetics, protein biochemistry, and immunology used but not explicitly described in this disclosure and these Examples are amply reported in the scientific literature and are well within the ability of those skilled in the art.

Example 1

This Example describes the construction of vectors containing the RSV F gene.

Figure 1 shows a restriction map of the gene encoding the F protein of Respiratory Syncytial Virus and Figure 2 shows the nucleotide sequence of the gene encoding the full-length RSV F protein (SEQ ID No: 1) and the deduced amino acid sequence (SEQ ID No: 2). Figure 3 shows the gene encoding the secreted RSV F protein (SEQ ID No: 3) and the deduced amino acid sequence (SEQ ID No: 4).

A set of four plasmid DNA constructs were made (as shown schematically in Figures 4 to 7) in which cDNA

encoding the RSV-F was subcloned downstream of the immediate-early promoter, enhancer and intron A sequences of human cytomegalovirus (CMV) and upstream of the bovine growth hormone (BGH) poly-A site. The 1.6 Kb SspI-PstI fragment containing the promoter, enhancer and intron A sequences of CMV Towne strain were initially derived from plasmid pRL43a obtained from Dr. G.S. Hayward of Johns Hopkins University (ref. 20) and subcloned between *Eco*RV and *Pst*I sites of pBluescript II SK +/- (Stratagene). For the construction of plasmids expressing the secretory form of the F protein (pXL1 and pXL2 in Figs. 4 and 5), the 1.6 Kb *Eco*RI-*Bam*HI fragment containing the truncated form of the F cDNA originally cloned from a clinical isolate belonging to subgroup A was excised from pRSVF (ref. 18 and WO 93/14207) and subcloned between *Eco*RI and *Bam*HI sites of pSG5 (Strategene, ref. 14). Either the 1.6 kb EcoRI-BamHI fragment or the 2.2 kb ClaI-BamHI fragment was then excised from the pSG5 construct, filled-in with Klenow and subcloned at the SmaI site of the pBluescript II SK +/- construct containing the promoter and intron A sequences. The 0.6 kb ClaI-EcoRI fragment derived from pSG5 contained the intron II sequences from rabbit β-globin. Subsequently, the plasmids were digested with HindIII, filled-in with Klenow, and digested with XbaI to yield either a 3.2 or a 3.8 Kb fragment. These fragments were used to replace the 0.8 kb NruI-XbaI fragment containing the CMV promoter in pRc/CMV (Invitrogen), resulting in the final pXL1 and pXL2 constructs, respectively.

For the construction of plasmids expressing the full-length F protein (pXL3 and pXL4 - Figs. 6 and 7), the full length RSV F cDNA was excised as a 1.9 kb EcoRI fragment from a recombinant pBluescript M13-SK (Stratagene) containing the insert (ref. 18 and WO 93/14207) and subcloned at the EcoRI site of pSG5

(Stratagene). Either the 1.9 Kb EcoRI fragment or the 2.5 Kb ClaI-BamHI fragment was then excised from the pSG5 construct, filled-in with Klenow and subcloned at the SmaI site of the pBluescript II SK +/- construct containing the promoter and intron A sequences. The rest of the construction for pXL3 and pXL4 was identical to that for pXL1 and pXL2, as described above. Therefore, except for the CMV promoter and intron A sequences, the rest of the vector components in pXL1-4 were derived from plasmid pRc/CMV. Plasmids pXL1 and pXL2 were made to express a truncated/secretory form of the F protein which carried stop codons resulting in a C-terminal deletion of 48 amino acids including the transmembrane (TM) and the C-terminal cytosolic tail as compared to the intact molecule. In contrast, pXL3 and pXL4 were made to express the intact membrane-attached form of the RSV F molecule containing the TM and the cytosolic C-terminal tail. The rationale for the presence of the intron II sequences in pXL2 and pXL4 was that this intron was reported to mediate the correct splicing of RNAs. Since mRNA for the RSV-F has been suspected to have a tendency towards aberrant splicing, the presence of the intron II sequences might help to overcome this. All four plasmid constructs were confirmed by DNA sequencing analysis.

Plasmid DNA was purified using plasmid mega kits from Qiagen (Chatsworth, CA, USA) according to the manufacturer's instructions.

Example 2

This Example describes the immunization of mice. Mice are susceptible to infection by RSV as described in ref. 16.

For intramuscular (i.m) immunization, the anterior tibialis anterior muscles of groups of 9 BALB/c mice (male, 6-8 week old) (Jackson Lab., Bar Harbor, ME, USA) were bilaterally injected with 2 x 50 μg (1 μg/μL in

PBS) of pXL1-4, respectively. Five days prior to DNA injection, the muscles were treated with 2 x 50 µL (10 µM in PBS) of cardiotoxin (Latoxan, France). Pretreatment of the muscles with cardiotoxin has been reported to increase DNA uptake and to enhance the subsequent immune responses by the intramuscular route (ref. 24). These animals were similarly boosted a month later. Mice in the control group were immunized with a placebo plasmid containing identical vector backbone sequences without the RSV F gene according to the same schedule. For intradermal (i.d.) immunization, 100 µg of pXL2 (2 µg/µL in PBS) were injected into the skin 1-2 cm distal from the tall base. The animals were similarly boosted a month later.

Seventy-five days after the second immunization, mice were challenged intranasally with $10^{5.4}$ plaque forming units (pfu) of mouse-adapted RSV, A2 subtype (obtained from Dr. P. Wyde, Baylor College of Medicine, Houston, TE, USA). Lungs were aseptically removed 4 days later, weighed and homogenized in 2 mL of complete culture medium. The number of pfu in lung homogenates was determined in duplicates as previously described (ref. 19) using vaccine quality Vero cells. These data were subjected to statistic analysis using SigmaStat (Jandel Scientific Software, Guelph, Ont. Canada).

Sera obtained from immunized mice were analyzed for anti-RSV F antibody titres (IgG, IgG1 and IgG2a, respectively) by enzyme-linked immunosorbent assay (ELISA) and for RSV-specific plaque-reduction titres. ELISA were performed using 96-well plates coated with immunoaffinity purified RSV F protein (50 ng/mL) and 2-fold serial dilutions of immune sera. A goat anti-mouse IgG antibody conjugated to alkaline phosphatase (Jackson ImmunoRes., Mississauga, Ont., Canada) was used as secondary antibody. For the measurement of IgG1 and

IgG2a antibody titres, the secondary antibodies used were monospecific sheep anti-mouse IgG1 (Serotec, Toronto, Ont., Canada) and rat anti-mouse IgG2a (Zymed, San Francisco, CA, USA) antibodies conjugated to alkaline phosphatase, respectively. Plaque reduction titres were determined according to Prince et al (ref. 19) using vaccine quality Vero cells. Four-fold serial dilutions of immune sera were incubated with 50 pfu of RSV, Long strain (ATCC) in culture medium at 37°C for 1 hr in the presence of 5% CO_2. Vero cells were then infected with the mixture. Plaques were fixed with 80% methanol and developed 5 days later using a mouse anti-RSV-F monoclonal IgG1 antibody and donkey antimouse IgG antibody conjugated to peroxidase (Jackson ImmunoRes., Mississauga, Ont. Canada). The RSV-specific plaque reduction titre was defined as the dilution of serum sample yielding 60% reduction in the number of plaques. Both ELISA and plaque reduction assays were performed in duplicates and data are expressed as the means of two determinations. These data were subjected to statistic analysis using SigmaStat (Jandel Scientific Software, Guelph, Ont. Canada).

To examine the induction of RSV-specific CTL following DNA immunization, spleens from 2 immunized mice were removed to prepare single cell suspensions which were pooled. Splenocytes were incubated at 2.5 x 10^6 cells/mL in complete RPMI medium containing 10 U/mL murine interleukin 2 (IL-2) with γ-irradiated (3,000 rads) syngeneic splenocytes (2.5 x 10^6 cells/mL) infected with 1 $TCID_{50}$/cell RSV (Long strain) for 2 hr. The source of murine IL-2 was supernatant of a mouse cell line constitutively secreting a high level of IL-2 obtained from Dr. H. Karasuyama of Basel Institute for Immunology (ref. 20). CTL activity was tested 5 days following the *in vitro* re-stimulation in a standard 4 hr chromium release assay. Target cells were 5 ^{51}Cr-

labelled uninfected BALB/c fibroblasts (BC cells) and persistently RSV-infected BCH14 fibroblasts, respectively. Washed responder cells were incubated with 2 x 10^3 target cells at varying effector to target ratios in 200 µL in 96-well V-bottomed tissue-culture plates for 4 hr at 37°C. Spontaneous and total chromium releases were determined by incubating target cells with either medium or 2.5% Triton-X 100 in the absence of responder lymphocytes. Percentage specific chromium release was calculated as (counts-spontaneous counts)/(total counts-spontaneous counts) X 100. Tests were performed in triplicates and data are expressed as the means of three determinations. For antibody blocking studies in CTL assays, the effector cells were incubated for 1 hr with 10 µg/ml final of purified mAb to CD4 (GK1.5) (ref. 21) or mAb against murine CD8 (53-6.7) (ref. 22) before adding chromium labelled BC or BCH4 cells. To determine the effect of anti-class I MHC antibodies on CTL killing, the chromium labelled target cells BC or BCH4 were incubated with 20 µL of culture supernate of hybridoma that secretes a mAb that recognizes K^d and D^d of class I MHC (34-1-2S) (ref. 23) prior to the addition of effector cells.

Example 3

This Example describes the immunogenicity and protection by polynucleotide immunization by the intramuscular route.

To characterize the antibody responses following i.m. DNA administration, immune sera were analyzed for anti-RSV F IgG antibody titre by ELISA and for RSV-specific plaque reduction titre, respectively. All four plasmid constructs were found to be immunogenic. Sera obtained from mice immunized with pXL1-4 demonstrated significant anti-RSV F IgG titres and RSV-specific plaque reduction titres as compared to the placebo group

(Table 1 below) ($P<0.0061$ and <0.0001, respectively, Mann-Whitney Test). However, there is no significant difference in either anti-RSV F IgG titre or RSV-specific plaque reduction titre among mice immunized with either pXL1, pXL2, pXL3 or pXL4.

To evaluate the protective ability of pXL1-4 against primary RSV infection of the lower respiratory tract, immunized mice were challenged intranasally with mouse-adapted RSV and viral lung titres post challenge were assessed. All four plasmid constructs were found to protect animals against RSV infection. A significant reduction in the viral lung titre was observed in mice immunized with pXL1-4 as compared to the placebo group ($P<0.0001$, Mann-Whitney Test). However, varying degrees of protection were observed depending on the plasmid. In particular, PXL1 was more protective than pXL3 ($P=0.00109$, Mann-Whitney Test), and pXL4 more than pXL3 ($P=0.00125$), whereas only pXL2 induced complete protection. This conclusion was confirmed by another analysis with number of fully protected mice as end point (Fisher Exact Test). Constructs pXL1, pXL2 or pXL4 conferred a higher degree of protection than pXL3 ($P<0.004$, Fisher Exact Test) which was not more effective than placebo. Only pXL2 conferred full protection in all immunized mice.

The above statistical analysis revealed that PXL1 conferred more significant protection than pXL3. The former expresses the truncated and secretory form and the latter the intact membrane anchored form of the RSV F protein. Furthermore, pXL4 was shown to be more protective than pXL3. The difference between these two constructs is the presence of the intron II sequence in pXL4. Construct pXL2 which expresses the secretory form of the RSV-F in the context of the intron II sequence was the only plasmid that confers complete protection in all immunized mice.

Example 4

This Example describes the influence of the route of administration of pXL2 on its immunogenicity and protective ability.

The i.m. and i.d. routes of DNA administration were compared for immunogenicity in terms of anti-RSV F antibody titres and RSV-specific plaque reduction titres. Analyses of the immune sera (Table 2 below) revealed that the i.d. route of DNA administration was as immunogenic as the i.m. route as judged by anti-RSV F IgG and IgG1 antibody responses as well as RSV-specific plaque reduction titres. However, only the i.m. route induced significant anti-RSV F IgG2a antibody responses, whereas the IgG2a isotype titre was negligible when the i.d. route was used. The i.m. and i.d. routes were also compared with respect to the induction of RSV-specific CTL. Significant RSV-specific CTL activity was detected in mice immunized intramuscularly. In contrast, the cellular response was significantly lower in mice inoculated intradermally (Table 3 below). In spite of these differences, protection against primary RSV infection of the lower respiratory tract was observed in both groups of mice immunized via either route (Table 4 below). The CTL induced by RSV-F DNA are classical CD8+ class I restricted CTL. The target cells, BCH4 fibroblasts express class I MHC only and do not express class II MHC. Further, prior incubation of BCH4 target cells with anti class-I MHC antibodies significantly blocked the lytic activity of RSV-F DNA induced CTL line. While anti-CD8 antibody could partially block lysis of BCH4 cells, antibody to CD4 molecule had no effect at all (Table 5 below). Lack of total blocking by mAb to CD8 could either be due to CTL being CD8 independent (meaning that even though they are CD8+ CTL, their TCR has enough affinity for class I MHC+peptide and it does not require CD8 interaction with the alpha 3

of class I MHC) or the amount of antibody used in these experiments was limiting. There was no detectable lysis of YAC-1 (NK sensitive target) cells (data not shown).

Example 5

This Example describes immunization studies in cotton rats using pXL2.

The immune response of cotton rats to DNA immunization was analyzed by the protocol shown in Table 6 below. On day -5, 40 cotton rats were randomly selected and divided into 8 groups of 5. Cotton rats in groups 1 and 7 were inoculated intramuscularly (i.m.) into the tiberlia anteria (TA) muscles bilaterally with cardiotoxin (1.0 μM). On day -1, the cotton rats in group 8 were inoculated in the TA muscles with bupivacaine (0.25%). On day 0, several animals in each group were bled to determine levels of RSV-specific antibodies in the serum of the test animals prior to administration of vaccines. All of the animals were then inoculated i.m. or intradermally (i.d.) with 200 μg of plasmid DNA, placebo (non-RSV-specific DNA), 100 median cotton rat infectious doses (CRID50; positive control) of RSV, or of formalin inactivated RSV prepared in Hep-2 tissue culture cells and adjuvanted in alum. Forty-four days later the cotton rats in groups 1 & 7 were reinoculated with cardiotoxin in the TA muscles. Four days later (48 days after priming with vaccine), the animals in group 8 were reinoculated with bupivacains in the TA muscle of the right leg. The next day, (seven weeks after priming with vaccine) all of the animals were bled and all, except those in the group given live RSV, were boosted with the same material and doses used on day 0. 29 days later, each cotton rat was bled and then challenged intranasally (i.n.) with 100 CRID50 RSV A2 grown in Hep-2 tissue culture cells. Four days after this virus challenge (day +88) all of the cotton rats were killed and their lungs removed. One

lobe from each set of lungs was fixed in formalin and then processed for histologic evaluation of pulmonary histopathology. The remaining lobes of lung will be assessed for the presence and levels of RSV. Each of the sera collected on days 0, 49 and 78 were tested for RSV-neutralizing activity, anti-RSV fusion activity and RSV-specific ELISA antibody.

The RSV neutralizing titres on day +49 and +78 are shown in Tables 7(a) below and 7(b) below respectively. As can be seen from the results shown in Table 7(a), on day +49 the animals immunized with live RSV and DNA immunization had substantial RSV serum neutralizing titres. The animals immunized with formalin-inactivated RSV had a neutralizing titre equivalent to the placebo group on day +49 but following boosting titres by day +78 had reached 5.8 ($log_{10}/0.05$). Boosting had no significant effect upon animals immunized with live RSV or by i.m. plasmid immunization.

RSV titres in nasal washes (upper respiratory tract) on day +82 are shown in Table 8 below. RSV titres in the lungs (lower respiratory tract) on day +82 are shown in Table 9 below. All of the vaccines provided protection against lung infection but under these conditions, only live virus provided total protection against upper respiratory tract infection.

The lungs from the cotton rats were examined histologically for pulmonary histopathology and the results are shown in Table 10 below. With the exception of lung sections obtained from Group 9 which were essentially free of inflammatory cells or evidence of inflammation, and those from Group 3, which exhibited the maximal pulmonary pathology seen in this study, all of the sections of lung obtained from the other groups looked familiar, i.e. scattered inflammatory cells were present in most fields, and there was some thickening of septae. These are evidence of mild inflammatory

diseases. Large numbers of inflammatory cells and other evidence of inflammation were present in sections of lung from Group 3 (in which formalin-inactivated [FI] RSV vaccine was given prior to virus challenge). This result indicated that immunization with plasmid DNA expressing the RSV F protein does not result in pulmonary histopathology different from the placebo, whereas FI-RSV caused more severe pathology.

Example 6

This Example describes the determination of local lung cytokine expression profile in mice imunized with pXL2 after RSV challenge.

Balb/C mice were immunized at 0 and 6 weeks with 100 μg of pXL2, prepared as described in Example 1, and challenged with RSV i.n. at 10 weeks. Control animals were immunized with FI-RSV and live RSV and challenged with RSV according to the same protocol. Four days post viral challenge, lungs were removed from immunized mice and immediately frozen in liquid nitrogen. Total RNA was prepared from lungs homogenized in TRIzol/β-mercaptoethanol by chloroform extraction and isopropanol precipitation. Reverse transcriptase-polymerase chain reaction (RT-PCR) was then carried out on the RNA samples using either IL-4, IL-5 or IFN-γ specific primers from Clone Tech. The amplified products were then liquid-hybridized to cytokine-specific ^{32}P-labeled probes from Clone Tech, resolved on 5% polyacrylamide gels and quantitated by scanning of the radioactive signals in the gels. Three mouse lungs were removed from each treatment group and analyzed for lung cytokine expression for a minimum of two times. The data is presented in Figure 9 and represents the means and standard deviations of these determinations.

As may be seen from the data presented in Figure 9:

1. Immunization with live RSV intranasally (i.n.) resulted in a balanced cytokine profile (IFN-γ, IL-4 and IL-5), whereas that with FI-RSV intramuscularly (i.m.) resulted in a Th2 predominance (elevated IL-4 and IL-5). These results are similar to what were reported in the literature.

2. Immunization with pXL2 containing the secretary (sec.) form of FI via either the i.m. or intradermal (i.d.) route gave rise to a balanced cytokine profile similar to that with live RSV immunization.

3. The magnitude of the cytokine responses with i.m. and i.d. immunication using pXL2 expressing a secretory form of the protein in significantly higher than that with live RSV immunization.

Example 7

This Example describes the construction of a plasmid vector encoding the RSV F protein and containing the 5' UTR and signal peptide of Herpes Simplex Virus I (HSV I)gD.

Plasmid p82M35B was prepared following the scheme shown in Figure 10. Plasmid pVR1012 (Vical) (Figure 11; SEQ ID No: 6) containing the CMV promoter, intron A, and the BGH poly A sequences, was linearized with restriction enzyme Pst I and made blunt ended with T4 DNA polymerase. The rabbit β-globin intron II sequence was retrieved from plasmid pSG5 (Stratagene; ref. 14) by Cla I and Eco RI digestion, and the 0.6 kb fragment was isolated and made blunt ended by treatment with Klenow fragment polymerase. The rabbit β-globin intron II fragment was then ligated to the Pst I/blunt ended VR1012 plasmid (Fig. 10). This vector was then restricted with Eco RV and dephosphorylated.

The secreted form of RSV F was isolated from plasmid pXL2 (Example 1; Fig. 5) by digestion with Sal I, made blunt end by treatment with Klenow fragment polymerase, then restricted with Kpn I to produce a 5' Kpn I, 3'blunt ended fragment. The HSV gD sequence was synthesized as a synthetic oligonucleotide having the DNA (SEQ ID No: 7) and derived amino acid (SEQ ID No: 8) sequence shown in Figure 12.

The gD oligonucleotide has a 5' blunt end and 3' Kpn I recognition sequence. A three-way ligation was performed with the isolated RSV F fragment, gD oligo and the VR1012 plasmid, to produce plasmid p82M35B (Fig. 10).

Example 8

This Example illustrates the expression and secretion of RSV F protein *in vitro*.

BHK cells were transfected with either p82M35B, its counterpart containing the autologous RSV F signal peptide (pXL2) or the vector backbone alone (placebo) using Lipofectin (Gtibco/BRL). Forty-eight hours post transfection, supernatant fractions were recovered and subjected to RSV F protein quantification using a F-specific enzyme-linked immunoabsorbent assay (ELISA). Three independent transfection assays were performed for each vector.

ELISAs were performed using one affinity-purified mouse monoclonal anti-RSV F antibody (2 μg/ml) as the capturing reagent and another biotinolated monoclonal anti-RSV F antibody (0.1 μg/ml) as the detection reagent. Horseradish peroxidase-labelled avidin (Pierce) was subsequently used. The RSV F standard protein used was purified from detergent-lysates of cultured virus by immunoaffinity chromatography.

Table 11 (below) shows the results obtained. As seen in Table II, compared to placebo, both p82M35B and pXL2 mediated significant F protein expression/secretion

from the BHK cells 48 hours post transfection. Furthermore, a markedly higher level of the F protein was consistently detected in the supernatant fraction of p82M35B-transfected BHK cells than that of pXL2-transfected cells, representing a 5.4-fold improvement over the latter. These results indicate that replacement of the coding sequence for the autologous RSV F signal peptide with that for the 5'UTR and signal peptide of HSV I gD significantly enhanced F protein expression/secretion *in vitro*.

Example 9

This Example illustrates immunogenicity studies carried out using p82M35B.

Tibialis anterior muscles of BALB/c mice (male, 6 to 8 weeks old) (Jackson Lab., Bar Harbor, ME, USA) were bilaterally injected with 2 x 50 µg (1 µg/µL in PBS) of p82M35B, pXL2 or the vector backbone alone (placebo). In some groups, 5 days prior to DNA injection, the muscles were treated with 2 x 50 µL (10 µM in PBS) of cardiotoxin (Latoxan, France) to increase DNA uptake and enhance immune responses as reported by Davis et al., (ref. 24). The animals were boosted with the same dose of plasmid DNA 6 weeks later. Mice in the positive control group were immunized intranasally (i.n.) with 10^6 plaque forming units (pfu) of a clinical RSV strain of the A2 subtype grown in Hep2 cells kindly provided by Dr. B. Graham (ref. 16).

Antisera obtained from immunized mice were analyzed for anti-RSV F IgG antibody titres using specific ELISA and for RSV-specific plaque-reduction titres. ELISAs were performed using 96-well plates coated with immunoaffinity-purified RSV F protein (50 ng/mL) and 2-fold serial dilutions of immune sera. A goat anti-mouse IgG antibody conjugated to alkaline phosphatase (Jackson ImmunoRes., Mississauga, Ont., Canada) was used as secondary antibody. Plaque reduction titres were

determined according to Prince et al. (ref. 19) using vaccine-quality Vero cells. Four-fold serial dilutions of immune sera were incubated with 50 pfu of the RSV Long strain (ATCC) in culture medium at 37°C for 1 hr in the presence of 5% CO_2 and the mixtures were used to infect Vero cells. Plaques were fixed with 80% methanol and developed 5 days later using a mouse anti-RSV F monoclonal IgG1 antibody and donkey anti-mouse IgG antibody conjugated to peroxidase (Jackson ImmunoRes. Mississauga, Ont.). The RSV-specific plaque reduction titre was defined as the dilution of serum sample yielding 60% reduction in plaque number. Both ELISAs and plaque reduction assays were performed in duplicate and data are expressed as the means of two determinations.

The results of these studies are set forth in Table 12 below. For the induction of serum antibody responses (Table 12), p82M35B is effective without the need of cardiotoxin pretreatment under the DNA dose and immunization regimen used, resulting in anti-F IgG titre of 7.2 ± 1.1 (log_2 titre/100) and RSV-specific plaque reduction titre of 11.8 ± 0.9 (log_2) after two immunizations. In contrast, the antibody titres elicited by pXL2 in the absence of the cardiotoxin pretreatment were significantly lower (IgG titre of 2.9 ± 2.3 and plaque reduction titre of 8.2 ± 1.9). However, serum antibody responses elicited by pXL2 were significantly improved with the cardiotoxin pretreatment step (IgG titre of 7.4 ± 1.1 and plaque reduction titre of 10.5 ± 0.8). The placebo was unable to elicit a detectable serum antibody response in the absence or presence of the cardiotoxin pretreatment step.

This trend was extendible to results of the protection study (Table 12). Vector p82M35B conferred full protection against RSV infection of lungs in the absence of the cardiotoxin pretreatment. In contrast,

pXL2 only conferred partial protection under the same conditions. However, full protection was achieved with the pXL2 vector when cardiotoxin pretreatment step was included in the immunization regimen. No protection was observed with the placebo with or without the cardiotoxin pretreatment step.

These results show the replacement of the coding sequence for the autologous RSV F signal peptide with that for the 5'UTR and signal peptide of HSV I gD resulted in significant enhancement in not only F protein expression/secretion assessed *in vitro* (Example 8), but also immunogenicity to the F protein as well as protective ability against RSV infection assessed in the mouse model.

SUMMARY OF THE DISCLOSURE

In summary of this disclosure, the present invention provides certain novel vectors containing genes encoding an RSV F proteins, methods of immunization using such vectors and methods of diagnosis using such vectors. Modifications are possible within the scope of this invention.

Table 1: Immunogenic and Protective Abilities of pXL1-4 Mice via the i.m. Route

Plasmid DNA Immunogen	No. Mice	Mean Anti-RSV F ELISA Titre(IgG)* (Log_2/100±SD)	Mean Plaque Reduction Titre* (Log_4±SD)	Post RSV Challenge: Mean Virus Lung Titre# (pfu/g lung) (Log_{10} ±SD)	Post RSV Challenge: No. Fully Protected Mice**
pXL1	8	3.00±1.85	3.74±0.98	0.72±0.99	5
pXL2	9	5.78±1.72	4.82±0.51	0.00±0.00	9
pXL3	8	3.75±2.05	4.59±1.16	2.77±0.72	0
pXL4	9	5.44±1.13	5.18±0.43	0.66±1.00	6
Placebo**	12	0.58±2.89	0.18±0.62	3.92±0.27	0

* These sets of data from sera obtained 1 week prior to the viral challenge
\# Detection sensitivity of the assay was $10^{1.96}$ pfu/g lung.
** The term, fully protected mice, refers to animals with no detectable RSV in lungs post challenge.

Table 2. Immunogenicity of pXL2 in Mice*

Route	No. Mice	Mean Anti-RSV F ELISA Titre (Log_2/100 + SD)			Mean Plaque Reduction Titre (Log_4 ± SD)
		IgG	IgG1	IgG2a	
i.m	8	7.63±0.92	4.25±1.91	4.38±1.92	4.18±0.88
i.d.	7	7.00±1.00	5.00±1.00	0.14±0.38	3.65±0.59
Placebo(i.m.)	9	0.50±0.51	0.00±0.00	0.00±0.00	0.18±0.50

* These sets of data are from sera obtained 1 week prior to the viral challenge.

Table 3. Induction of RSV-specific CTL Following DNA Immunization*

Route	E:T Ratio	% Specific Lysis	
		BC	BCH4
i.m.	200:1	23.3	100.6
	100:1	17.0	62.4
	50:1	19.9	64.1
	25:1	22.3	46.4
i.d.	100:1	20.9	26.1
	50:1	21.7	19.1
	25:1	7.1	7.0
	12.5:1	2.8	2.3

* These set of data were obtained from immunized mice immediately prior to RSV challenge.

Table 4. Immunoprotective Ability of pXL2 in Mice

Route	No. Mice	Post RSV Challenge	
		Mean Virus Lung Titre* (pfu/g lung)	No. Fully Protected Mice#
i.m.	8	0.00±0.00	8
i.d.	7	0.43±1.13	6
Placebo (i.m.)	9	4.30±022	0

* Detection sensitivity of the assay was $10^{1.69}$ pfu/g lung.
\# The term, fully protected mice, refers to animals with no detectable RSV in lungs post challenge.

Table 5. RSV specific CTL included by i.m. DNA immunization are class I restricted CTL

E:T Ratio	BCH4	BCH4+anti-CD4	BCH4+anti-CD8	BCH4+anti-class I MHC
100:1	52.03	54.3	39.4	8.6
50:1	44.4	47.2	27.4	6.2
25:1	28.6	26.3	14.8	1
12.5:1	18.2	15	8	-2.7

Table 6

Group	Antigen	RSV-specific dose	Inoc. route	Pretreatment/ Adjuvant	Day 0	Day 49	Day 78	Day 88
1	Placebo	0	I.M.	Cardiotoxin	Prebleed, several cotton rats per group; prime all animals	Bleed all animals; boost all except those in group 2	Challenge with RSV A2 I.N. after bleeding all	Harv. animals and do histologic evaluation, pulmonary virus titers, antibodies
2	Live RSV	100 CRID50	I.N.	None				
3	FI-RSV		I.M.	Alum				
5	pXL2	200 μg	I.M.	None				
6	pXL2	200 μg	I.D.	None				
7	pXL2	200 μg	I.M.	Cardiotoxin				
8	pXL2	200 μg	I.M.	Bupivacaine				

Table 7(a). RSV Serum Neutralizing Titers on Day 49

Group	Antigen	RSV-specific dose	Inoc. route	Nt. antibody titer (log_2/0.05 ml) in CR no.				Mean titer log2/ 0.05	Stand. Dev.
				1	2	3	4		
1	Placebo	0	I.M.	4	3	2	2	2.75	1.0
2	Live RSV	100 CRID50	I.N.	9	9	9	9	9	0.0
3	FI-RSV		I.M.	0	4	2	2	2.0	1.6
5	pXL2	200 μg	I.M.	9	8	8	7	8.0	0.8
6	pXL2	200 μg	I.D.	5	2	5	5	4.3	1.5
7	pXL2	200 μg	I.M.	8	8	9	9	8.5	0.6
8	pXL2	200 μg	I.M.	8	9	6	6	7.3	1.5

Table 7(b). RSV Serum Neutralizing Titers on Day 78

Group	Antigen	RSV-specific dose	Inoc. route	Nt. antibody titer (log_2/0.05 ml) in CR no.				Mean titer log_2/0.05	Stand. Dev.
				1	2	3	4		
1	Placebo	0	I.M.	3	2	4	Died	3.0	1.0
2	Live RSV	100 CRID50	I.N.	8	9	8	9	8.5	0.6
3	FI-RSV		I.M.	8	4	6	5	5.8	1.7
5	pXL2	200 μg	I.M.	7	8	8	8	7.8	0.5
6	pXL2	200 μg	I.D.	8	6	6	Died	6.7	1.2
7	pXL2	200 μg	I.M.	8	9	9	8	8.7	0.6
8	pXL2	200 μg	I.M.	8	7	9	9	8.3	1.0

Table 8. RSV Titers in Nasal Washes on Day 82

Group	Antigen	RSV-specific dose	Inoc. route	RSV titer (log_{10}/0.05 ml) in cotton rat no.				Mean titer log_{10}/0.05	Stand. Dev.
				1	2	3	4		
1	Placebo	0	I.M.	3.4	3.3	3.3	Died	3.3	0.1
2	Live RSV	100 CRID50	I.N.	0	0	0	0	0.0	0.0
3	FI-RSV		I.M.	0	0	2.8	0	0.7	1.4
5	pXL2	200 µg	I.M.	3.3	2.3	3.3	2.3	2.8	0.6
6	pXL2	200 µg	I.D.	N.D.	N.D.	N.D.	Died	N.D.	N.D.
7	pXL2	200 µg	I.M.	2.3	0	0	3.2	1.4	1.6
8	pXL2	200 µg	I.M.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

N.D. = non-determined

Table 9. Titers in Lungs on Day 82

Group	Antigen	RSV-specific dose	Inoc. route	RSV titer (log_{10}/g lung) in cotton rat no.				Mean titer log_{10}/0.05	Stand. Dev.
				1	2	3	4		
1	Placebo	0	I.M.	4.7	4.2	3.7	Died	4.2	0.5
2	Live RSV	100 CRID50	I.N.	0	0	0	0	0.0	0.0
3	FI-RSV	10^5 PFU	I.M.	0	0	0	0	0.0	0.0
5	pXL2	200 μg	I.M.	0	2.2	0	0	0.6	1.1
6	pXL2	200 μg	I.D.	0	2.2	2.7	3.2	2.0	N.D.
7	pXL2	200 μg	I.M.	0	0	0	0	0.0	0.0
8	pXL2	200 μg	I.M.	0	0	0	0	0.0	N.D.

N.D. = non-determined

Table 10. **Summary of Histopathology Results Seen in Sections of Cotton Rat Lung.**

Group	Treatment	Major Observations & Comments
1.	Placebo + RSV	Scattered individual and groups of macrophages and polymorphonuclear neutrophiles (PMN) in all fields. Overt thickening of septae. Occasional pyknotic cells seen. Overall: mild to moderate inflammation.
2.	Live RSV	Isolated macrophages seen in most fields. Scattered PMN. Overall: minimal inflammation
3.	FI-RSV + RSV	Virtually every field contains numerous mononuclear cells & PMN. Pyknotic cells and debris common. Thickened septae. Evidence of exacerbated disease.
5.	Plasmid + RSV	Isolated macrophages seen in most fields. Occasional PMN seen. Very similar to live virus group.
6.	Plasmid i.d. + RSV	Isolated macrophages seen in most fields. Occasional PMN seen.
7.	Plasmid + CT + RSV	Isolated mononuclear cells and PMN seen in most fields.
8.	Plasmid + Biv + RSV	Scattered mononuclear cells and PMN seen in most fields.
9.	Normal CR Lung	Few leukocytes evidence. Airy, open appearance. Thin septae.

CT = carditoxin
Biv = bupivacaine

Table 11. Expression/Secretion of the RSV F protein from BHK cells
(48 hr post transfection)

Plasmid Construct	F Protein Secretion (mean ± S.D.) (ng/mL)	Magnitude of Improvement
Placebo	0.0 ± 0.0	
p82M35B	32.1 ± 2.06	5.4 x (over pXL2)
pXL2	5.9 ± 0.6	

Table 12. Immunoprotective Ability of DNA-F in BALB/c Mice

Immunogen	Anti-F IgG Titre Log2 (titre/100) 10 weeks	RSV-Specific Plaque Reduction Titre (Log_2 titre)	Mean Virus Lung Titre* (pfu/g lung) (Log_2 10 ± SD)	No. Fully protected # / No. Immunized
Placebo (i.m.)	0.0 ± 0.0	0.0 ± 0.0	4.3 ± 0.5	0/6
p82M35B (i.m.)	7.2 ± 1.1	11.8 ± 0.9	0.0 ± 0.0	6/6
pXL2 (i.m.)	2.9 ± 2.3	8.2 ± 1.9	2.9 ± 1.7	1/6
pXL2 + cardiotoxin	7.4 ± 1.1	10.5 ± 0.8	0.0 ± 0.0	6/6
RSV (i.n.)	8.5 ± 2.7	12.4 ± 0.7	0.0 ± 0.0	6/6

* Sensitivity of assay: $10^{1.69}$ pfu/g lung.

The term, fully protected mice, refers to animals with no detectable RSV in the lungs 4 days post viral challenge.

REFERENCES

1. McIntosh K., Canock, R.M. In: Fields BN, Knipe, DM, editors. Virology. New York: Raven Press: 1990: 1045-1072.

2. Katz SL., In: New Vaccine Development establishing priorities. Vol. 1. Washington: National Academic Press: 1985: 397-409.

3. Wertz GW, Sullender WM., Biotechnology 1992; 20: 151-176.

4. Johnson et al., J. Virol 1987, 61: 3163-3166.

5. Pemberton et al., J. Gen Virol. 1987, 68: 2177-2182.

6. Crowe, J.E., Vaccine 1995, 13: 415-421.

7. WO 90/11092

8. WO 94/21797

9. Ulmer, Current Opinion, Invest Drugs, 1993, 2: 983-989.

10. Tang et al., Nature 1992, 356: 152-154.

11. Furth et al. Analytical Biochemistry, 1992, 205: 365-368.

12. Pizzorno et al., J. Virol. 1988, 62: 1167-1179.

13. Chapman, B.S.; Thayer, R.M.; Vincent, K.A. and Haigwood, N.L., Nucl. Acids. Res. 1991, 19: 3979-3986.

14. Green, S. Isseman, I., and Sheer, E., Nucl. Acids. Res. 1988, 16: 369.

15. Breathnack, R. and Harris, B.A., Nucl. Acids Res. 1983, 11: 7119-7136.

16. Graham, B.S.; Perkins M.D.; Wright, P.F. and Karzon, D.T. J. Mod. Virol. 1988 26: 153-162.

17. Nabel, G.J. 1993, Proc. Natl. Acad. Sci. USA 90: 11307-11311.

18. Du, R.P et al. 1994., Biotechnology 12: 813-818.

19. Prince, G.A. et al, 1978. Ame. J. Pathol. 93: 771-790.

20. Karasuyama & Melchers, Eur. J. Immunol. 18, 97-104, 1988.

21. Wilde, David et al. 1983. J. Immunol. 131: 2178-2183.

22. Ledbetter, J.A., Rouse R., Micklem, H. 1980, J. Exp. Med. 152: 280-295

23. Ozato Kerko et al. 1982, Transplantation 34: 113-118.

24. Davis et al., Vaccine 1994, 12: 1503-1509

CLAIMS

What we claim is:

1. An immunogenic composition for *in vivo* administration to a host for the generation in the host of a protective immune response to RSV F protein, comprising a non-replicating vector comprising:

a first nucleotide sequence encoding an RSV F protein or a RSV F protein fragment that generates antibodies and/or cytotoxic T-lymphocytes (CTLs) that specifically react with RSV F protein;

a promoter sequence operatively coupled to the first nucleotide sequence for expression of said RSV F protein in the host, and

a second nucleotide sequence located between said first nucleotide sequence and said promoter sequence to enhance the immunoprotective ability of said RSV F protein when expressed *in vivo* from said vector in a host; and

a pharmaceutically-acceptable carrier therefor.

2. The composition of claim 1 wherein said first nucleotide sequence encodes a full-length RSV F protein.

3. The composition of claim 1 wherein said first nucleotide sequence encodes a RSV F protein from which the transmembrane region is absent.

4. The composition of claim 1 wherein said first nucleotide sequence encodes a RSV F protein fragment lacking a transmembrane coding region.

5. The composition of claim 1 wherein said promoter sequence is an immediate early cytomegalovirus promoter.

6. The composition of claim 1 wherein said second nucleotide sequence comprises a pair of splice sites to prevent aberrant mRNA splicing, whereby substantially all RNA transcribed from the vector encodes an RSV F protein.

7. The composition of claim 6 wherein said second nucleotide sequence is located between said first nucleotide sequence and said promoter sequence.

8. The composition of claim 7 wherein said second nucleotide sequence is that of rabbit β-globin intron II.

9. The composition of claim 1 wherein said first nucleotide sequence encodes a RSV F protein fragment lacking an autologous RSV F signal peptide sequence and includes a sequence encoding a heterologous signal peptide which enhances the level of expression of RSV F protein.

10. The composition of claim 9 wherein said first nucleotide sequence encoding a signal peptide encodes HSV I gD.

11. The composition of claim 10 wherein said first nucleotide sequence encodes a RSV F protein fragment lacking a transmembrane coding region.

12. The composition of claim 1 wherein said non-replicating vector is plasmid pXL2 as shown in Figure 5.

13. The composition of claim 1 wherein said non-replicating vector is plasmid pXL4 as shown in Figure 7.

14. The composition of claim 1 wherein said non-replicating vector is plasmid p82M35B as shown in Figure 10.

15. A method of immunizing a host against disease caused by infection with respiratory syncytial virus (RSV), which comprises administering to said host an effective amount of a non-replicating vector comprising a first nucleotide sequence encoding an RSV F protein or a RSV F protein fragment that generates antibodies and/or cytotoxic T-lymphocytes (CTLs) that specifically react with RSV F protein and a promoter sequence operatively coupled to said first nucleotide sequence for expression of said RSV F protein in said host.

16. The method of claim 15, wherein the first nucleotide sequence encodes a full-length RSV F protein.

17. The method of claim 15 , wherein said first nucleotide sequence encodes a RSV F protein fragment lacking a transmembrane region.

18. The method of claim 15 wherein the first nucleotide sequence encodes a RSV F protein fragment lacking an autologous RSV F signal peptide sequence and includes a sequence encoding a heterologous signal peptide which enhances the level of expression of RSV F protein.

19. The composition of claim 18 wherein the first nucleotide sequence encodes a signal peptide encodes HSV I gD.

20. The composition of claim 19 wherein the first nucleotide sequence encodes a RSV F protein fragment lacking a transmembrane coding region.

21. The method of claim 15 wherein said host is a human.

22. The method of claim 21 wherein said promoter sequence is an immediate early cytomegalovirus promoter.

23. The method of claim 15 wherein said non-replicating vector further comprises a second nucleotide sequence located adjacent said first nucleotide sequence to enhance the immunoprotective ability of said RSV F protein when expressed *in vivo* from said vector in said host.

24. The method of claim 23 wherein said promoter sequence is an immediate early cytomegalovirus promoter.

25. The method of claim 24 wherein said second nucleotide sequence comprises a pair of splice sites to prevent aberrant mRNA splicing, whereby substantially all mRNA transcribed from the vector encodes an RSV F protein.

26. The method of claim 25 wherein said second nucleotide sequence is located between said first nucleotide sequence and said promoter sequence.

27. The method of claim 26 wherein said second nucleotide sequence is that of rabbit β-globulin intron II.

28. The method of claim 23 wherein said vector is pXL2 as shown in Figure 5.

29. The method of claim 23 wherein said vector is pXL4 as shown in Figure 7.

30. The method of claim 23 wherein said vector is p82M35B as shown in Figure 10.

31. A method of using a gene encoding an RSV F protein or a RSV protein fragment that generates antibodies and/or cytotoxic T-lymphocytes (CTLs) that specifically react with RSV F protein to produce an immune response in a host, which comprises:

isolating said gene;

operatively linking said gene to at least one control sequence to produce a non-replicating vector, said control sequence directing expression of said RSV F protein when said vector is introduced into a host to produce an immune response to said RSV F protein or fragment thereof; and

introducing said vector into the host.

32. The method of claim 31 wherein said gene encoding an RSV F protein encodes an RSV F protein lacking the transmembrane region.

33. The method of claim 32 wherein said at least one control sequence comprises the immediate early cytomegalovirus promoter.

34. The method of claims 31 wherein said gene coding an RSV F protion encodes a RSV F protein fragment lacking an autologous RSV F signal peptide sequence and includes a sequence encoding a heterologous signal peptide which enhance the level of expression of RSV F protein.

35. The method of claims 34 wherein said gene coding an RSV F protion encoding a signal peptide encodes HSV I gD.

36. The method of claim 35 wherein said gene encoding a RSV F protein encodes a RSV F protein fragment lacking a transmembrane coding region.

37. The method of claim 36 wherein said at least one control sequence comprises the immediately early cytomegalovirus promoter.

38. The method of claim 37 including the step of:

operatively linking said gene to an immunoprotective enhancing sequence to produce an enhanced immunoprotection to said RSV F protein in said host.

39. The method of claim 38 wherein said immunoprotective enhancing sequence is introduced into said vector between said control sequence and said gene.

40. The method of claim 39 wherein said immunoprotection enhancing sequence comprises a pair of splice sites to prevent aberrant mRNA splicing whereby substantially cell transcribed mRNA encodes an RSV F protein.

41. The method of claim 40 wherein said immunoprotection enhancing sequence is that of rabbit β-globin intron II.

42. The method of claim 31 wherein said gene is contained within a plasmid vector selected from the group consisting of pXL2, pXL4 and p82M35B.

43. A method of producing a vaccine for protection of a host against disease caused by infection with respiratory syncytial virus (RSV), which comprises:

isolating a first nucleotide sequence encoding an RSV F protein or a RSV F protein fragment that generates antibodies and/or cytotoxic T-lymphocytes (CTLs) that specifically react with RSV F protein;

operatively linking said first nucleotide sequence to at least one control sequence to produce a non-replicating vector, the control sequence directing expression of said RSV F protein when introduced into a

host to produce an immune response to said RSV F protein;

operatively linking said first nucleotide sequence to a second nucleotide sequence to enhance the immunoprotective ability of said RSV F protein when expressed *in vivo* from the vector in a host, and

formulating said vector as a vaccine for *in vivo* administration.

44. The method of claim 43 wherein said non-replicating vector is a plasmid vector selected from the group consisting of pXL2, pXL4 and p82M35B.

45. A vaccine produced by the method of claim 43.

46. A method of producing a vaccine for protection of a host against disease caused by infection with respiratory syncytial virus (RSV), which comprises:

isolating a first nucleotide sequence encoding an RSV F protein from which the transmembrane region is absent;

operatively linking said first nucleotide sequence to at least one control sequence to produce a non-replicating vector, the control sequence directing expression of said RSV F protein when introduced into a host to produce an immune response to said RSV F protein; and

formulating said vector as a vaccine for *in vivo* administration.

47. The method of claim 46 wherein said vector is a plasmid vector selected from group consisting of pXL2 and p82M35B.

48. A vaccine produced by the method of claim 46.

49. A method of determining the presence of a respiratory syncytial virus (RSV) F protein in a sample, comprising the steps of:

(a) immunizing a host with a non-replicating vector comprising a first nucleotide sequence encoding an RSV F protein or a RSV F protein

fragment that generates antibodies that specifically react with RSV F protein and a promoter sequence operatively coupled to said first nucleotide sequence for expression of said RSV F protein in said host to produce antibodies specific for the RSV F protein;

(b) isolating the RSV F protein specific antibodies;

(c) contacting the sample with the isolated antibodies to produce complexes comprising any RSV F protein present in the sample and said isolated RSV F protein-specific antibodies; and

(d) determining production of the complexes.

50. The method of claim 49 wherein said vector is a plasmid vector selected from the group consisting of pXL1, pXL2, pXL3, pXL4 and p82M35B.

51. A diagnostic kit for detecting the presence of an RSV F protein in a sample, comprising:

(a) a non-replicating vector comprising a first nucleotide sequence encoding an RSV F protein or a RSV F protein fragment that generates antibodies that specifically react with RSV F protein and a promoter sequence operatively coupled to said first nucleotide sequence for expression of said RSV F protein in a host immunized therewith to produce antibodies specific for the RSV F protein;

(b) isolation means to isolate said RSV F protein-specific antibodies;

(c) contacting means to contact the isolated RSV F specific antibodies with the sample to produce a complex comprising any RSV F protein present in the sample and RSV F protein specific antibodies, and

(d) identifying to determine production of the complex.

52. The diagnostic kit of claim 51 wherein said vector is a plasmid vector selected from the group consisting of pXL1, pXL2, pXL3, pXL4 and p82M35B.

53. A method for producing antibodies specific for an F protein of RSV comprising:

(a) immunizing a host with an effective amount of a non-replicating vector comprising a first nucleotide sequence encoding an RSV F protein lacking a transmembrane region and a promoter sequence operatively coupled to said first nucleotide sequence for expression of said RSV F protein in said host to produce antibodies specific for the F protein; and

(b) isolating the antibodies from the host.

54. A method of producing monoclonal antibodies specific for an F protein of RSV comprising the steps of:

(a) constructing a non-replicating vector comprising a first nucleotide sequence encoding an RSV F protein and a promoter sequence operatively coupled to said first nucleotide sequence for expression of said RSV F protein; and, optionally,

a second nucleotide sequence located adjacent said first nucleotide sequence to enhance the immunoprotective ability of said RSV F protein when expressed *in vivo* from said vector in a host.

(b) administering the vector to at least one mouse to produce at least one immunized mouse;

(c) removing B-lymphocytes from the at least one immunized mouse;

(d) fusing the B-lymphocytes from the at least one immunized mouse with myeloma cells, thereby producing hybridomas;

(e) cloning the hybridomas;

(f) selecting clones which produce anti-F protein antibody;

(g) culturing the anti-F protein antibody-producing clones; and

(h) isolating anti-F protein monoclonal antibodies from the cultures.

ABSTRACT OF THE DISCLOSURE

Non-replicating vectors containing a nucleotide sequence coding for an F protein of respiratory syncytial virus (RSV) and a promoter for such sequence, preferably a cytomegalovirus promoter, are described for *in vivo* immunization. The nucleotide sequence encloding the RSV F protein may lack a sequence encoding the homologous signal peptide but possessing a heterologous signal peptide enhancing RSV F protein expression. Such non-replicating vectors, including plasmids, also may contain a further nucleotide sequence located adjacent to the RSV F protein encoding sequence to enhance the immunoprotective ability of the RSV F protein when expressed *in vivo*. Such non-replicating vectors may be used to immunize a host against disease caused by infection with RSV, including a human host, by administration thereto, and may be formulated as immunogenic compositions with pharmaceutically-acceptable carriers for such purpose. Such vectors also may be used to produce antibodies for detection of RSV infection in a sample.

Docket No.
1038-922 MIS:jb

Declaration and Power of Attorney For Patent Application

English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

NUCLEIC ACID RESPIRATORY SYNCYTIAL VIRUS VACCINES

the specification of which

(check one)

☒ is attached hereto.

☐ was filed on _____ as United States Application No. or PCT International Application Number _____

and was amended on _____
(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Not Claimed
(Number)	(Country)	(Day/Month/Year Filed)	☐
(Number)	(Country)	(Day/Month/Year Filed)	☐
(Number)	(Country)	(Day/Month/Year Filed)	☐

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

08/896,500	**July-18-1997**	**Pending**
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
08/659,939	**June-7-1996**	**Patented**
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
08/476,397	**June 7, 1995**	**Pending**
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*

Michael I. Stewart (24,973)

Send Correspondence to: **Sim & McBurney**
6th Floor, 330 University Avenue
Toronto, Ontario
Canada, M5G 1R7.

Direct Telephone Calls to: *(name and telephone number)*
(416) 595-1155

Full name of sole or first inventor **Xiaomao Li**
Sole or first inventor's signature Date
Residence **Thornhill, Ontario, Canada**
Citizenship **Canadian**
Post Office Address **106 Glenmanor Way, Thornhill, Ontario, Canada, L4J 3E5.**

Full name of second inventor, if any **Mary E. Ewasyshyn**
Second inventor's signature Date
Residence **Willowdale, Ontario, Canada**
Citizenship **Canadian**
Post Office Address **Apt. 1506, 120 Torresdale Avenue, Willowdale, Ontario, Canada, M2R 3N7.**

Full name of third inventor, if any
Suryaprakash Sambhara

Third inventor's signature Date

Residence
Markham, Ontario, Canada

Citizenship
Canadian

Post Office Address
50 Harness Circle, Markham, Ontario, Canada, L3S 1Y1.

Full name of fourth inventor, if any
Michel H. Klein

Fourth inventor's signature Date

Residence
Willowdale, Ontario, Canada

Citizenship
Canadian

Post Office Address
16 Munro Boulevard, Willowdale, Ontario, Canada, M2P 1B9.

Full name of fifth inventor, if any

Fifth inventor's signature Date

Residence

Citizenship

Post Office Address

Full name of sixth inventor, if any

Sixth inventor's signature Date

Residence

Citizenship

Post Office Address

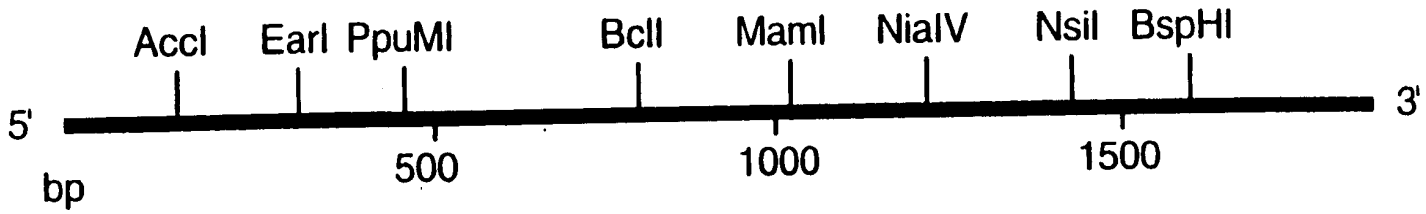

RESTRICTION MAP OF THE RSV F GENE
AccI
EarI
PpuMI
BclI
MamI
NiaIV
NsiI
BspHI
5'
3'
bp
500
1000
1500

FIG.1

FIG. 2A

NUCLEOTIDE SEQUENCE OF THE RSV F GENE.

SP

```
5'  MET GLU LEU PRO ILE LEU LYS ALA ASN ALA ILE THR THR ILE LEU ALA ALA VAL THR PHE
    ATGGAGTTGCCAATCCTCAAAGCAAATGCAATTACCACAATCCTCGCTGCAGTCACATTT
    TACCTCAACGGTTAGGAGTTTCGTTTACGTTAATGGTGTTAGGAGCGACGTCAGTGTAAA
              10        20        30        40        50        60

    CYS PHE ALA SER SER GLN ASN ILE THR GLU GLU PHE TYR GLN SER THR CYS SER ALA VAL
    TGCTTTGCTTCTAGTCAAAACATCACTGAAGAATTTTATCAATCAACATGCAGTGCAGTT
    ACGAAACGAAGATCAGTTTTGTAGTGACTTCTTAAAATAGTTAGTTGTACGTCACGTCAA
              70        80        90       100       110       120

    SER LYS GLY TYR LEU SER ALA LEU ARG THR GLY TRP TYR THR SER VAL ILE THR ILE GLU
    AGCAAAGGCTATCTTAGTGCTCTAAGAACTGGTTGGTATACTAGTGTTATAACTATAGAA
    TCGTTTCCGATAGAATCACGAGATTCTTGACCAACCATATGATCACAATATTGATATCTT
             130       140       150       160       170       180

    LEU SER ASN ILE LYS GLU ASN LYS CYS ASN GLY THR ASP ALA LYS VAL LYS LEU MET LYS
    TTAAGTAATATCAAGGAAAATAAGTGTAATGGAACAGATGCTAAGGTAAAATTGATGAAA
    AATTCATTATAGTTCCTTTTATTCACATTACCTTGTCTACGATTCCATTTTAACTACTTT
             190       200       210       220       230       240

    GLN GLU LEU ASP LYS TYR LYS ASN ALA VAL THR GLU LEU GLN LEU LEU MET GLN SER THR
    CAAGAATTAGATAAATATAAAAATGCTGTAACAGAATTGCAGTTGCTCATGCAAAGCACA
    GTTCTTAATCTATTTATATTTTTACGACATTGTCTTAACGTCAACGAGTACGTTTCGTGT
             250       260       270       280       290       300

    PRO ALA ALA ASN ASN ARG ALA ARG ARG GLU LEU PRO ARG PHE MET ASN TYR THR LEU ASN
    CCAGCAGCAAACAATCGAGCCAGAAGAGAACTACCAAGGTTTATGAATTATACACTCAAC
    GGTCGTCGTTTGTTAGCTCGGTCTTCTCTTGATGGTTCCAAATACTTAATATGTGAGTTG
             310       320       330       340       350       360
```

FIG. 2B

F2-F1CLEAVAGE SITE

ASN THR LYS LYS THR ASN VAL THR LEU SER LYS LYS ARG LYS ARG ARG↓PHE LEU GLY PHE
AATACCAAAAAAACCAATGTAACATTAAGCAAGAAAAGGAAAA8AAGATTTCTTGGTTTT
TTATGGTTTTTTTGGTTACATTGTAATTCGTTCTTTTCCTTTTCTTCTAAAGAACCAAAA
370 380 390 400 410 420

LEU LEU GLY VAL GLY SER ALA ILE ALA SER GLY ILE ALA VAL SER LYS VAL LEU HIS LEU
TTGTTAGGTGTTGGATCTGCAATCGCCAGTGGCATTGCTGTATCTAAGGTCCTGCACTTA
AACAATCCACAACCTAGACGTTAGCGGTCACCGTAACGACATAGATTCCAGGACGTGAAT
430 440 450 460 470 480

GLU GLY GLU VAL ASN LYS ILE LYS SER ALA LEU LEU SER THR ASN LYS ALA VAL VAL SER
GAAGGAGAAGTGAACAAGATCAAAAGTGCTCTACTATCCACAAACAAGGCCGTAGTCAGC
CTTCCTCTTCACTTGTTCTAGTTTTCACGAGATGATAGGTGTTTGTTCCGGCATCAGTCG
490 500 510 520 530 540

LEU SER ASN GLY VAL SER VAL LEU THR SER LYS VAL LEU ASP LEU LYS ASN TYR ILE ASP
TTATCAAATGGAGTTAGTGTCTTAACCAGCAAAGTGTTAGACCTCAAAAACTATATAGAT
AATAGTTTACCTCAATCACAGAATTGGTCGTTTCACAATCTGGAGTTTTTGATATATCTA
550 560 570 580 590 600

LYS GLN LEU LEU PRO ILE VAL ASN LYS GLN SER CYS ARG ILE SER ASN ILE GLU THR VAL
AAACAATTGTTACCTATTGTGAATAAGCAAAGCTGCAGAATATCAAATATAGAAACTGTG
TTTGTTAACAATGGATAACACTTATTCGTTTCGACGTCTTATAGTTTATATCTTTGACAC
610 620 630 640 650 660

ILE GLU PHE GLN HIS LYS ASN ASN ARG LEU LEU GLU ILE THR ARG GLU PHE SER VAL ASN
ATAGAGTTCCAACAAAAGAACAACAGACTACTAGAGATTACCAGGGAATTTAGTGTTAAT
TATCTCAAGGTTGTTTTCTTGTTGTCTGATGATCTCTAATGGTCCCTTAAATCACAATTA
670 680 690 700 710 720

ALA GLY VAL THR THR PRO VAL SER THR TYR MET LEU THR ASN SER GLU LEU LEU SER LEU
GCAGGTGTAACTACACCTGTAAGCACTTACATGTTAACTAATAGTGAATTATTGTCATTA
CGTCCACATTGATGTGGACATTCGTGAATGTACAATTGATTATCACTTAATAACAGTAAT
730 740 750 760 770 780

FIG. 2C

```
ILE ASN ASP MET PRO ILE THR ASN ASP GLN LYS LYS LEU MET SER ASN ASN VAL GLN ILE
ATCAATGATATGCCTATAACAAATGATCAGAAAAAGTTAATGTCCAACAATGTTCAAATA
TAGTTACTATACGGATATTGTTTACTAGTCTTTTTCAATTACAGGTTGTTACAAGTTTAT
         790       800       810       820       830       840

VAL ARG GLN GLN SER TYR SER ILE MET SER ILE ILE LYS GLU GLU VAL LEU ALA TYR VAL
GTTAGACAGCAAAGTTACTCTATCATGTCCATAATAAAAGAGGAAGTCTTAGCATATGTA
CAATCTGTCGTTTCAATGAGATAGTACAGGTATTATTTTCTCCTTCAGAATCGTATACAT
         850       860       870       880       890       900

VAL GLN LEU PRO LEU TYR GLY VAL ILE ASP THR PRO CYS TRP LYS LEU HIS THR SER PRO
GTACAATTACCACTATATGGTGTGATAGATACACCTTGTTGGAAATTACACACATCCCCT
CATGTTAATGGTGATATACCACACTATCTATGTGGAACAACCTTTAATGTGTGTAGGGGA
         910       920       930       940       950       960

LEU CYS THR THR ASN THR LYS GLU GLY SER ASN ILE CYS LEU THR ARG THR ASP ARG GLY
CTATGTACAACCAACACAAAAGAAGGGTCAAACATCTGTTTAACAAGAACTGACAGAGGA
GATACATGTTGGTTGTGTTTTCTTCCCAGTTTGTAGACAAATTGTTCTTGACTGTCTCCT
         970       980       990      1000      1010      1020

TRP TYR CYS ASP ASN ALA GLY SER VAL SER PHE PHE PRO GLN ALA GLU THR CYS LYS VAL
TGGTACTGTGACAATGCAGGATCAGTATCTTTCTTCCCACAAGCTGAAACATGTAAAGTT
ACCATGACACTGTTACGTCCTAGTCATAGAAAGAAGGGTGTTCGACTTTGTACATTTCAA
        1030      1040      1050      1060      1070      1080

GLN SER ASN ARG VAL PHE CYS ASP THR MET ASN SER LEU THR LEU PRO SER GLU VAL ASN
CAATCGAATCGAGTATTTTGTGACACAATGAACAGTTTAACATTACCAAGTGAAGTAAAT
GTTAGCTTAGCTCATAAAACACTGTGTTACTTGTCAAATTGTAATGGTTCACTTCATTTA
        1090      1100      1110      1120      1130      1140

LEU CYS ASN VAL ASP ILE PHE ASN PRO LYS TYR ASP CYS LYS ILE MET THR SER LYS THR
CTCTGCAATGTTGACATATTCAATCCCAAATATGATTGTAAAATTATGACTTCAAAAACA
GAGACGTTACAACTGTATAAGTTAGGGTTTATACTAACATTTTAATACTGAAGTTTTTGT
        1150      1160      1170      1180      1190      1200
```

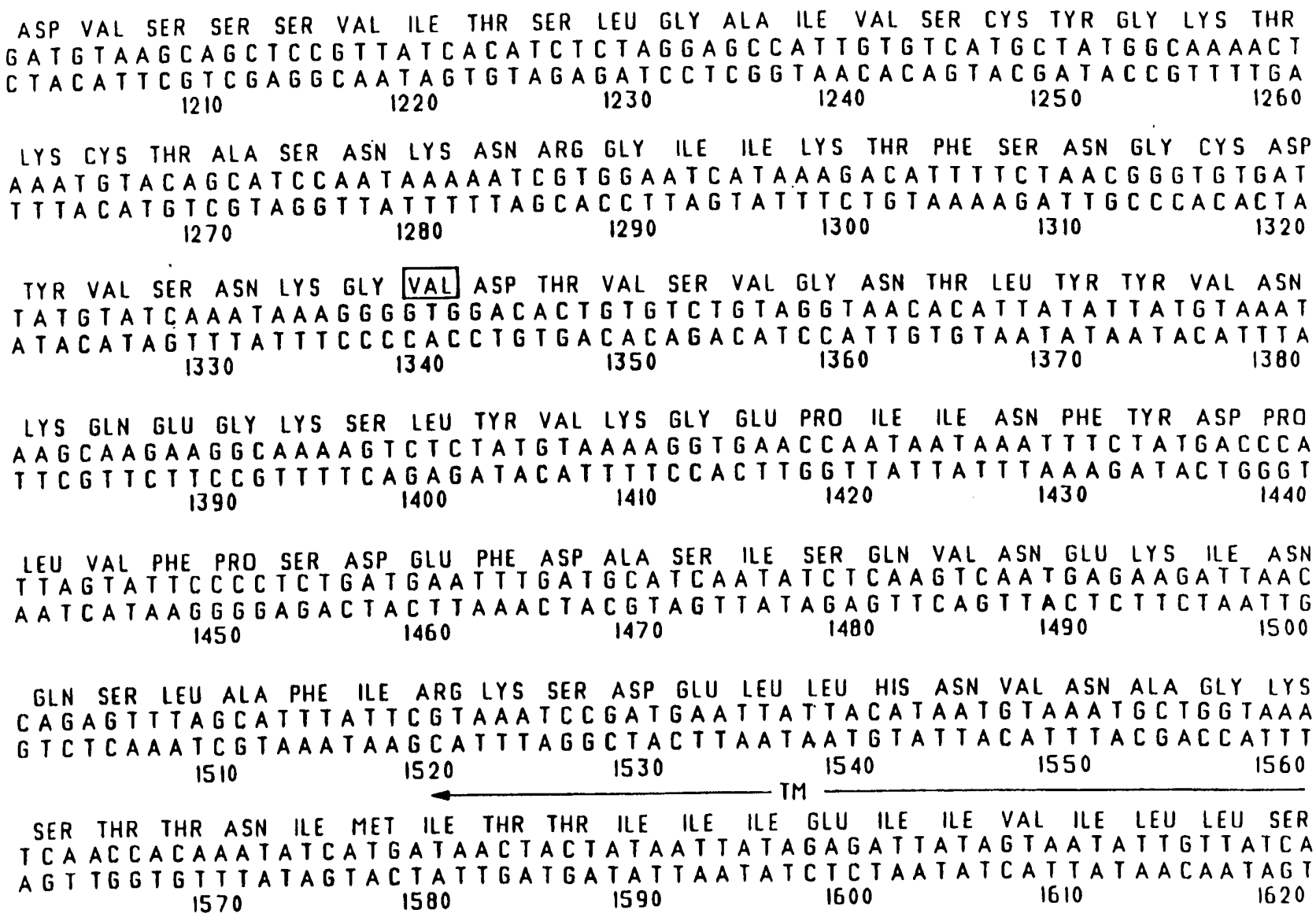
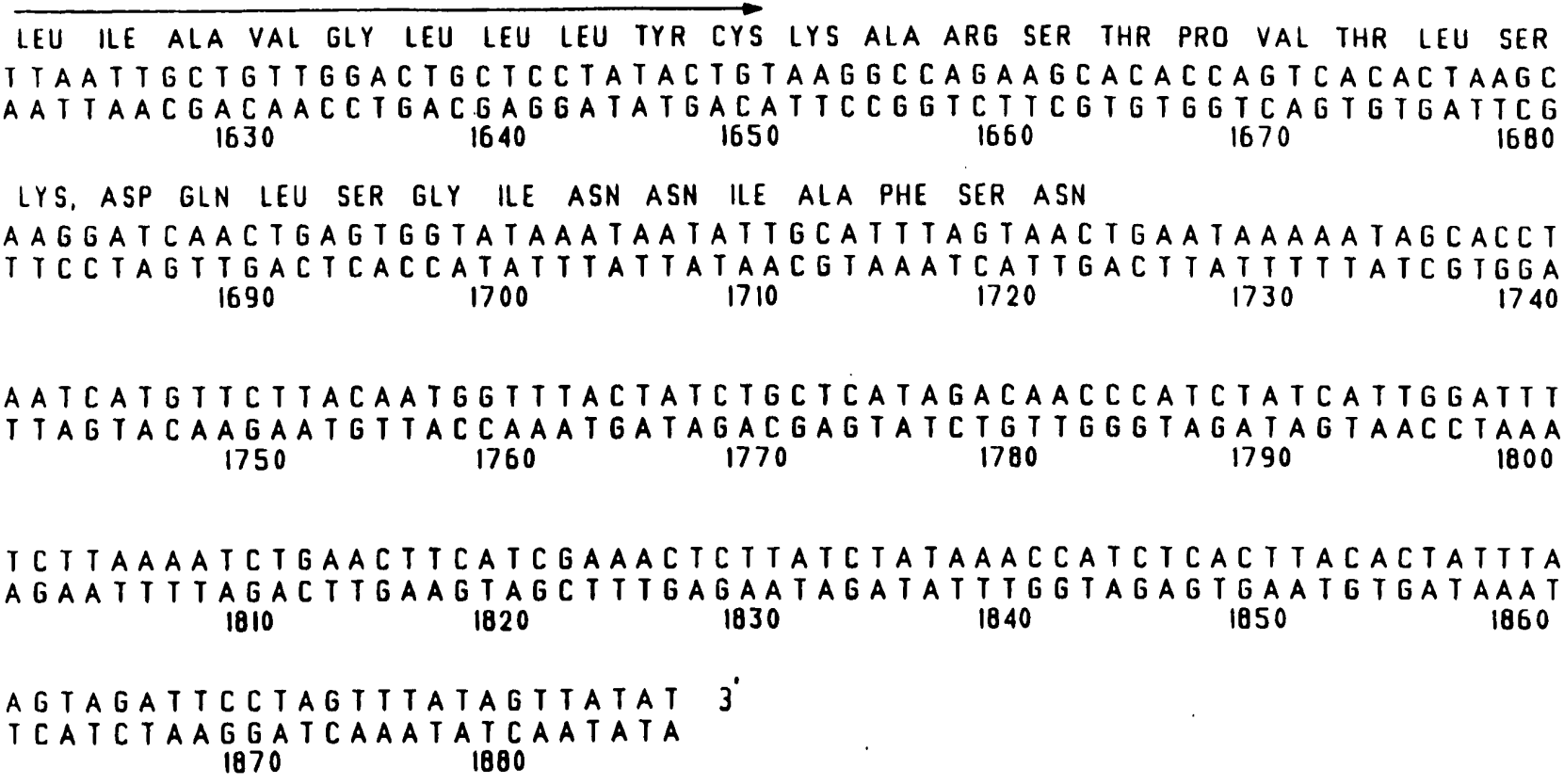

ASP VAL SER SER SER VAL ILE THR SER LEU GLY ALA ILE VAL SER CYS TYR GLY LYS THR
GATGTAAGCAGCTCCGTTATCACATCTCTAGGAGCCATTGTGTCATGCTATGGCAAAACT
CTACATTCGTCGAGGCAATAGTGTAGAGATCCTCGGTAACACAGTACGATACCGTTTTGA
1210 1220 1230 1240 1250 1260
LYS CYS THR ALA SER ASN LYS ASN ARG GLY ILE ILE LYS THR PHE SER ASN GLY CYS ASP
AAATGTACAGCATCCAATAAAAATCGTGGAATCATAAAGACATTTTCTAACGGGTGTGAT
TTTACATGTCGTAGGTTATTTTTAGCACCTTAGTATTTCTGTAAAAGATTGCCCACACTA
1270 1280 1290 1300 1310 1320
TYR VAL SER ASN LYS GLY VAL ASP THR VAL SER VAL GLY ASN THR LEU TYR TYR VAL ASN
TATGTATCAAATAAAGGGGTGGACACTGTGTCTGTAGGTAACACATTATATTATGTAAAT
ATACATAGTTTATTTCCCCACCTGTGACACAGACATCCATTGTGTAATATAATACATTTA
1330 1340 1350 1360 1370 1380
LYS GLN GLU GLY LYS SER LEU TYR VAL LYS GLY GLU PRO ILE ILE ASN PHE TYR ASP PRO
AAGCAAGAAGGCAAAAAGTCTCTATGTAAAAGGTGAACCAATAATAAATTTCTATGACCCA
TTCGTTCTTCCGTTTTCAGAGATACATTTTCCACTTGGTTATTATTTAAAGATACTGGGT
1390 1400 1410 1420 1430 1440
LEU VAL PHE PRO SER ASP GLU PHE ASP ALA SER ILE SER GLN VAL ASN GLU LYS ILE ASN
TTAGTATTCCCCTCTGATGAATTTGATGCATCAATATCTCAAGTCAATGAGAAGATTAAC
AATCATAAGGGGAGACTACTTAAACTACGTAGTTATAGAGTTCAGTTACTCTTCTAATTG
1450 1460 1470 1480 1490 1500
GLN SER LEU ALA PHE ILE ARG LYS SER ASP GLU LEU LEU HIS ASN VAL ASN ALA GLY LYS
CAGAGTTTAGCATTTATTCGTAAATCCGATGAATTATTACATAATGTAAATGCTGGTAAA
GTCTCAAATCGTAAATAAGCATTTAGGCTACTTAATAATGTATTACATTTACGACCATTT
1510 1520 1530 1540 1550 1560
TM
SER THR THR ASN ILE MET ILE THR THR ILE ILE ILE GLU ILE ILE VAL ILE LEU LEU SER
TCAACCACAAATATCATGATAACTACTATAATTATAGAGATTATAGTAATATTGTTATCA
AGTTGGTGTTTATAGTACTATTGATGATATTAATATCTCTAATATCATTATAACAATAGT
1570 1580 1590 1600 1610 1620

FIG. 2D



NUCLEOTIDE SEQUENCE OF THE RSV F GENE.THE cDNA SEQUENCE IS SHOWN IN THE PLUS (mRNA) STRAND SENSE IN THE 5' TO 3' DIRECTION.THE SIGNAL PEPTIDE (SP) AND THE TRANSMEMBRANE (TM) ANCHOR DOMAIN ARE UNDERLINED.THE PREDICTED F2-F1 CLEAVAGE SITE IS INDICATED BY THE ARROW (↓).

FIG. 2E

FIG. 3A

NUCLEOTIDE SEQUENCE OF THE RSV F GENE.

SP

5' MET GLU LEU PRO ILE LEU LYS ALA ASN ALA ILE THR THR ILE LEU ALA ALA VAL THR PHE
ATGGAGTTGCCAATCCTCAAAGCAAATGCAATTACCACAATCCTCGCTGCAGTCACATTT
TACCTCAACGGTTAGGAGTTTCGTTTACGTTAATGGTGTTAGGAGCGACGTCAGTGTAAA
10 20 30 40 50 60

CYS PHE ALA SER SER GLN ASN ILE THR GLU GLU PHE TYR GLN SER THR CYS SER ALA VAL
TGCTTTGCTTCTAGTCAAAACATCACTGAAGAATTTTATCAATCAACATGCAGTGCAGTT
ACGAAACGAAGATCAGTTTTGTAGTGACTTCTTAAAATAGTTAGTTGTACGTCACGTCAA
70 80 90 100 110 120

SER LYS GLY TYR LEU SER ALA LEU ARG THR GLY TRP TYR THR SER VAL ILE THR ILE GLU
AGCAAAGGCTATCTTAGTGCTCTAAGAACTGGTTGGTATACTAGTGTTATAACTATAGAA
TCGTTTCCGATAGAATCACGAGATTCTTGACCAACCATATGATCACAATATTGATATCTT
130 140 150 160 170 180

LEU SER ASN ILE LYS GLU ASN LYS CYS ASN GLY THR ASP ALA LYS VAL LYS LEU MET LYS
TTAAGTAATATCAAGGAAAATAAGTGTAATGGAACAGATGCTAAGGTAAAATTGATGAAA
AATTCATTATAGTTCCTTTTATTCACATTACCTTGTCTACGATTCCATTTTAACTACTTT
190 200 210 220 230 240

GLN GLU LEU ASP LYS TYR LYS ASN ALA VAL THR GLU LEU GLN LEU LEU MET GLN SER THR
CAAGAATTAGATAAATATAAAAATGCTGTAACAGAATTGCAGTTGCTCATGCAAAGCACA
GTTCTTAATCTATTTATATTTTTACGACATTGTCTTAACGTCAACGAGTACGTTTCGTGT
250 260 270 280 290 300

PRO ALA ALA ASN ASN ARG ALA ARG ARG GLU LEU PRO ARG PHE MET ASN TYR THR LEU ASN
CCAGCAGCAAACAATCGAGCCAGAAGAGAACTACCAAGGTTTATGAATTATACACTCAAC
GGTCGTCGTTTGTTAGCTCGGTCTTCTCTTGATGGTTCCAAATACTTAATATGTGAGTTG
310 320 330 340 350 360

FIG. 3B

F2-F1CLEAVAGE SITE

ASN THR LYS LYS THR ASN VAL THR LEU SER LYS LYS ARG LYS ARG ARG↓PHE LEU GLY PHE
AATACCAAAAAAACCAATGTAACATTAAGCAAGAAAAGGAAAA8AAGATTTCTTGGTTTT
TTATGGTTTTTTTGGTTACATTGTAATTCGTTCTTTTCCTTTTCTTCTAAAGAACCAAAA
370 380 390 400 410 420

LEU LEU GLY VAL GLY SER ALA ILE ALA SER GLY ILE ALA VAL SER LYS VAL LEU HIS LEU
TTGTTAGGTGTTGGATCTGCAATCGCCAGTGGCATTGCTGTATCTAAGGTCCTGCACTTA
AACAATCCACAACCTAGACGTTAGCGGTCACCGTAACGACATAGATTCCAGGACGTGAAT
430 440 450 460 470 480

GLU GLY GLU VAL ASN LYS ILE LYS SER ALA LEU LEU SER THR ASN LYS ALA VAL VAL SER
GAAGGAGAAGTGAACAAGATCAAAAGTGCTCTACTATCCACAAACAAGGCCGTAGTCACC
CTTCCTCTTCACTTGTTCTAGTTTTCACGAGATGATAGGTGTTTGTTCCGGCATCAGTCG
490 500 510 520 530 540

LEU SER ASN GLY VAL SER VAL LEU THR SER LYS VAL LEU ASP LEU LYS ASN TYR ILE ASP
TTATCAAATGGAGTTAGTGTCTTAACCAGCAAAGTGTTAGACCTCAAAAACTATATAGAT
AATAGTTTACCTCAATCACAGAATTGGTCGTTTCACAATCTGGAGTTTTTGATATATCTA
550 560 570 580 590 600

LYS GLN LEU LEU PRO ILE VAL ASN LYS GLN SER CYS ARG ILE SER ASN ILE GLU THR VAL
AAACAATTGTTACCTATTGTGAATAAGCAAAGCTGCAGAATATCAAATATAGAAACTGTG
TTTGTTAACAATGGATAACACTTATTCGTTTCGACGTCTTATAGTTTATATCTTTGACAC
610 620 630 640 650 660

ILE GLU PHE GLN HIS LYS ASN ASN ARG LEU LEU GLU ILE THR ARG GLU PHE SER VAL ASN
ATAGAGTTCCAACAAAAGAACAACAGACTACTAGAGATTACCAGGGAATTTAGTGTTAAT
TATCTCAAGGTTGTTTTCTTGTTGTCTGATGATCTCTAATGGTCCCTTAAATCACAATTA
670 680 690 700 710 720

ALA GLY VAL THR THR PRO VAL SER THR TYR MET LEU THR ASN SER GLU LEU LEU SER LEU
GCAGGTGTAACTACACCTGTAAGCACTTACATGTTAACTAATAGTGAATTATTGTCATTA
CGTCCACATTGATGTGGACATTCGTGAATGTACAATTGATTATCACTTAATAACAGTAAT
730 740 750 760 770 780

FIG. 3C

ILE ASN ASP MET PRO ILE THR ASN ASP GLN LYS LYS LEU MET SER ASN ASN VAL GLN ILE
ATCAATGATATGCCTATAACAAATGATCAGAAAAAGTTAATGTCCAACAATGTTCAAATA
TAGTTACTATACGGATATTGTTTACTAGTCTTTTTCAATTACAGGTTGTTACAAGTTTAT
790 800 810 820 830 840

VAL ARG GLN GLN SER TYR SER ILE MET SER ILE ILE LYS GLU GLU VAL LEU ALA TYR VAL
GTTAGACAGCAAAGTTACTCTATCATGTCCATAATAAAAGAGGAAGTCTTAGCATATGTA
CAATCTGTCGTTTCAATGAGATAGTACAGGTATTATTTTCTCCTTCAGAATCGTATACAT
850 860 870 880 890 900

VAL GLN LEU PRO LEU TYR GLY VAL ILE ASP THR PRO CYS TRP LYS LEU HIS THR SER PRO
GTACAATTACCACTATATGGTGTGATAGATACACCTTGTTGGAAATTACACACATCCCCT
CATGTTAATGGTGATATACCACACTATCTATGTGGAACAACCTTTAATGTGTGTAGGGGA
910 920 930 940 950 960

LEU CYS THR THR ASN THR LYS GLU GLY SER ASN ILE CYS LEU THR ARG THR ASP ARG GLY
CTATGTACAACCAACACAAAAGAAGGGTCAAACATCTGTTTAACAAGAACTGACAGAGGA
GATACATGTTGGTTGTGTTTTCTTCCCAGTTTGTAGACAAATTGTTCTTGACTGTCTCCT
970 980 990 1000 1010 1020

TRP TYR CYS ASP ASN ALA GLY SER VAL SER PHE PHE PRO GLN ALA GLU THR CYS LYS VAL
TGGTACTGTGACAATGCAGGATCAGTATCTTTCTTCCCACAAGCTGAAACATGTAAAGTT
ACCATGACACTGTTACGTCCTAGTCATAGAAAGAAGGGTGTTCGACTTTGTACATTTCAA
1030 1040 1050 1060 1070 1080

GLN SER ASN ARG VAL PHE CYS ASP THR MET ASN SER LEU THR LEU PRO SER GLU VAL ASN
CAATCGAATCGAGTATTTTGTGACACAATGAACAGTTTAACATTACCAAGTGAAGTAAAT
GTTAGCTTAGCTCATAAAACACTGTGTTACTTGTCAAATTGTAATGGTTCACTTCATTTA
1090 1100 1110 1120 1130 1140

LEU CYS ASN VAL ASP ILE PHE ASN PRO LYS TYR ASP CYS LYS ILE MET THR SER LYS THR
CTCTGCAATGTTGACATATTCAATCCCAAATATGATTGTAAAATTATGACTTCAAAAACA
GAGACGTTACAACTGTATAAGTTAGGGTTTATACTAACATTTTAATACTGAAGTTTTTGT
1150 1160 1170 1180 1190 1200

FIG. 3D

```
ASP VAL SER SER SER VAL ILE THR SER LEU GLY ALA ILE VAL SER CYS TYR GLY LYS THR
GATGTAAGCAGCTCCGTTATCACATCTCTAGGAGCCATTGTGTCATGCTATGGCAAAACT
CTACATTCGTCGAGGCAATAGTGTAGAGATCCTCGGTAACACAGTACGATACCGTTTTGA
        1210      1220      1230      1240      1250      1260

LYS CYS THR ALA SER ASN LYS ASN ARG GLY ILE ILE LYS THR PHE SER ASN GLY CYS ASP
AAATGTACAGCATCCAATAAAAATCGTGGAATCATAAAGACATTTTCTAACGGGTGTGAT
TTTACATGTCGTAGGTTATTTTTAGCACCTTAGTATTTCTGTAAAAGATTGCCCACACTA
        1270      1280      1290      1300      1310      1320

TYR VAL SER ASN LYS GLY VAL ASP THR VAL SER VAL GLY ASN THR LEU TYR TYR VAL ASN
TATGTATCAAATAAAGGGGTGGACACTGTGTCTGTAGGTAACACATTATATTATGTAAAT
ATACATAGTTTATTTCCCCACCTGTGACACAGACATCCATTGTGTAATATAATACATTTA
        1330      1340      1350      1360      1370      1380

LYS GLN GLU GLY LYS SER LEU TYR VAL LYS GLY GLU PRO ILE ILE ASN PHE TYR ASP PRO
AAGCAAGAAGGCAAAAGTCTCTATGTAAAAGGTGAACCAATAAJAAATTTCTATGACCCA
TTCGTTCTTCCGTTTTCAGAGATACATTTTCCACTTGGTTATTATTTAAAGATACTGGGT
        1390      1400      1410      1420      1430      1440

LEU VAL PHE PRO SER ASP GLU PHE ASP ALA SER ILE SER GLN VAL ASN GLU LYS ILE ASN
TTAGTATTCCCCTCTGATGAATTTGATGCATCAATATCTCAAGTCAATGAGAAGATTAAC
AATCATAAGGGGAGACTACTTAAACTACGTAGTTATAGAGTTCAGTTACTCTTCTAATTG
        1450      1460      1470      1480      1490      1500

GLN SER LEU ALA PHE ILE ARG LYS SER ASP GLU LEU LEU HIS ASN VAL ASN ALA GLY LYS
CAGAGTTTAGCATTTATTCGTAAATCCGATGAATTATTACATAATGTAAATGCTGGTAAA
GTCTCAAATCGTAAATAAGCATTTAGGCTACTTAATAATGTATTACATTTACGACCATTT
        1510      1520      1530      1540      1550      1560

SER THR THR ASN ILE MET Thr Stop Stop Stop Bam HI
TCAACCACAAATATCATGACTTGATAATGAGGATCC
AGTTGGTGTTTATAGTACTGAACTATTACTCCTAGG
        1570
```

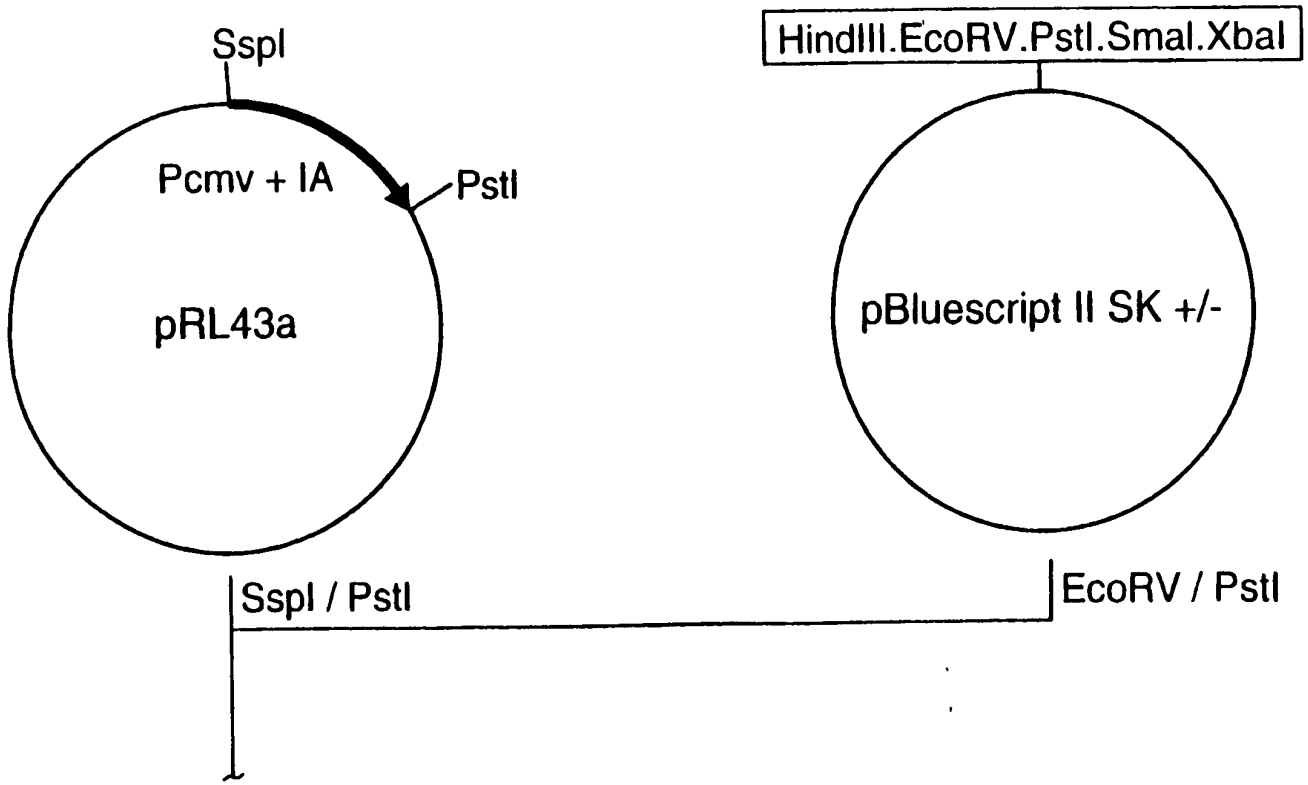

SspI
Pcmv + IA
PstI
pRL43a
HindIII.EcoRV.PstI.SmaI.XbaI
pBluescript II SK +/-
SspI / PstI
EcoRV / PstI

FIG.4A

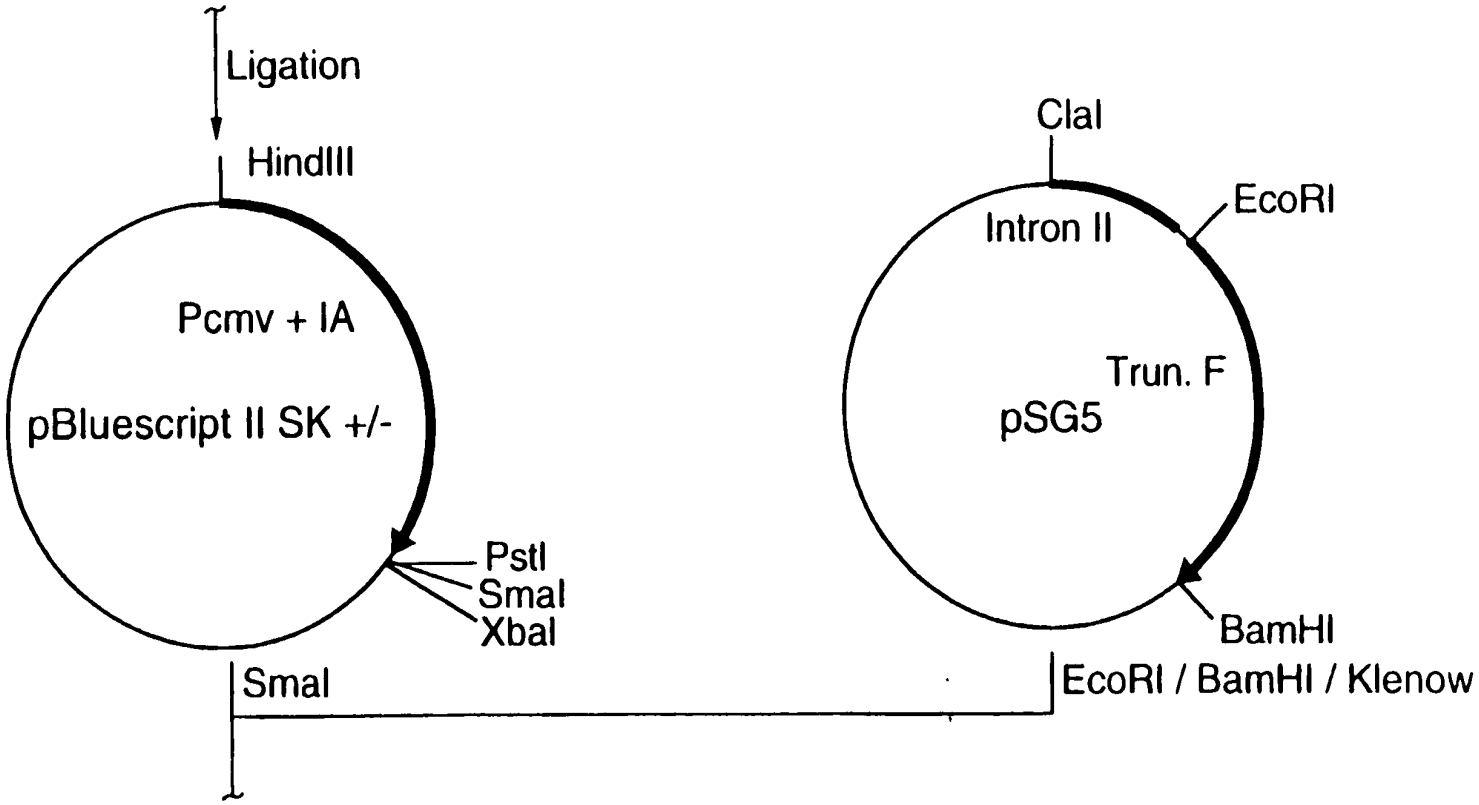

Ligation
HindIII
Pcmv + IA
pBluescript II SK +/-
PstI
SmaI
XbaI
SmaI
ClaI
EcoRI
Intron II
Trun. F
pSG5
BamHI
EcoRI / BamHI / Klenow

FIG.4B

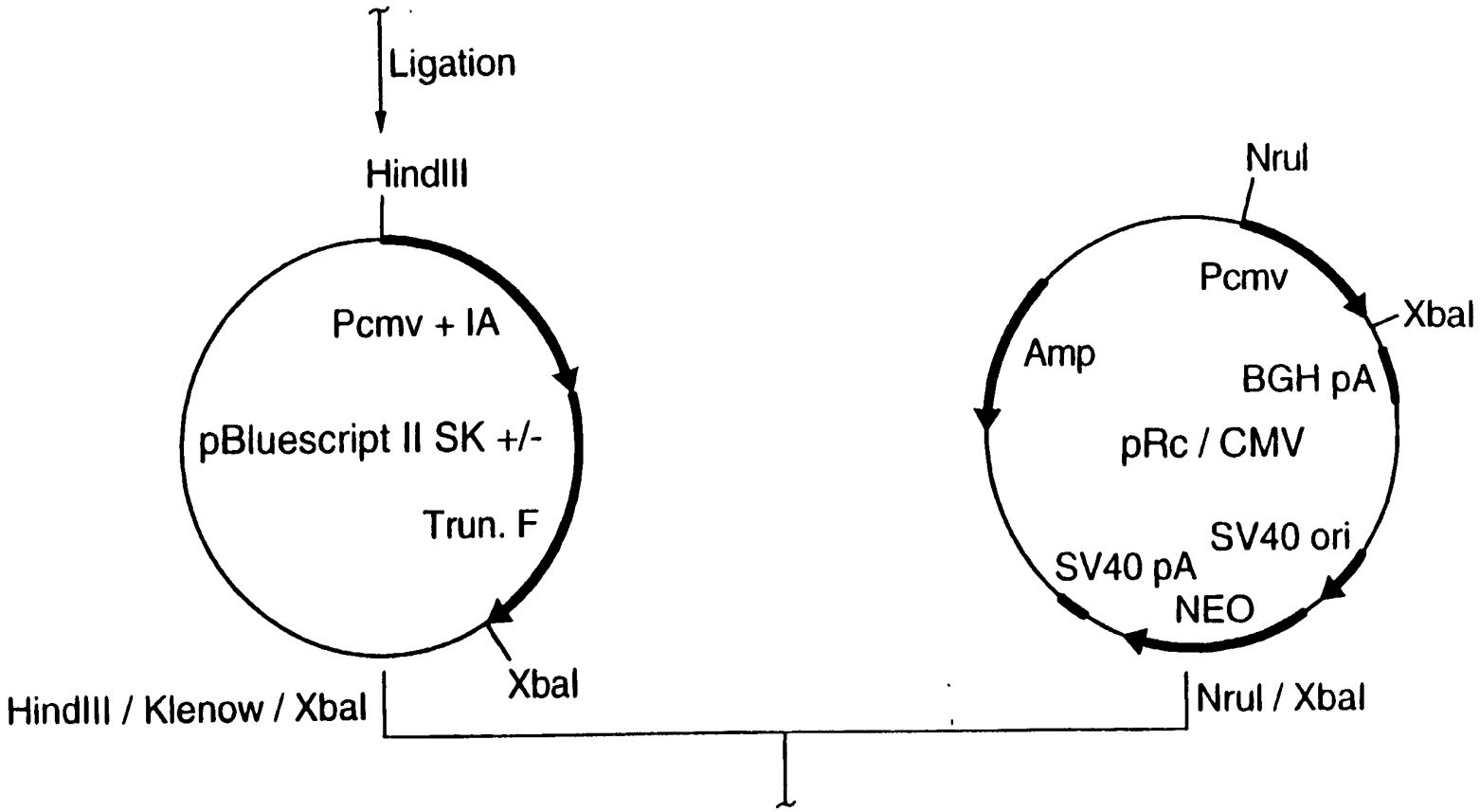

Ligation
HindIII
Pcmv + IA
pBluescript II SK +/-
Trun. F
XbaI
HindIII / Klenow / XbaI
NruI
Pcmv
XbaI
Amp
BGH pA
pRc / CMV
SV40 ori
SV40 pA
NEO
NruI / XbaI

FIG.4C

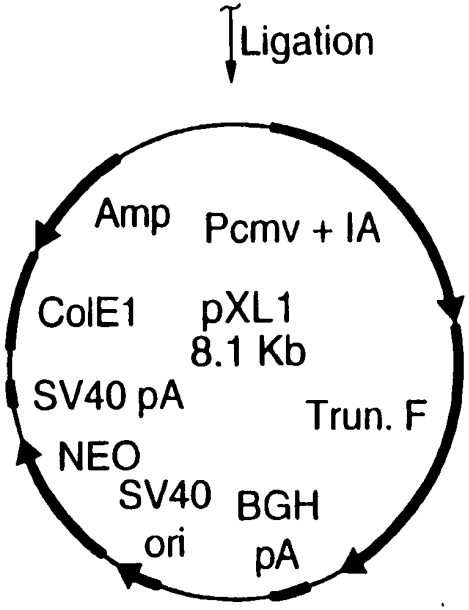

Ligation
Amp
Pcmv + IA
ColE1
pXL1
8.1 Kb
SV40 pA
Trun. F
NEO
SV40
ori
BGH
pA

FIG.4D

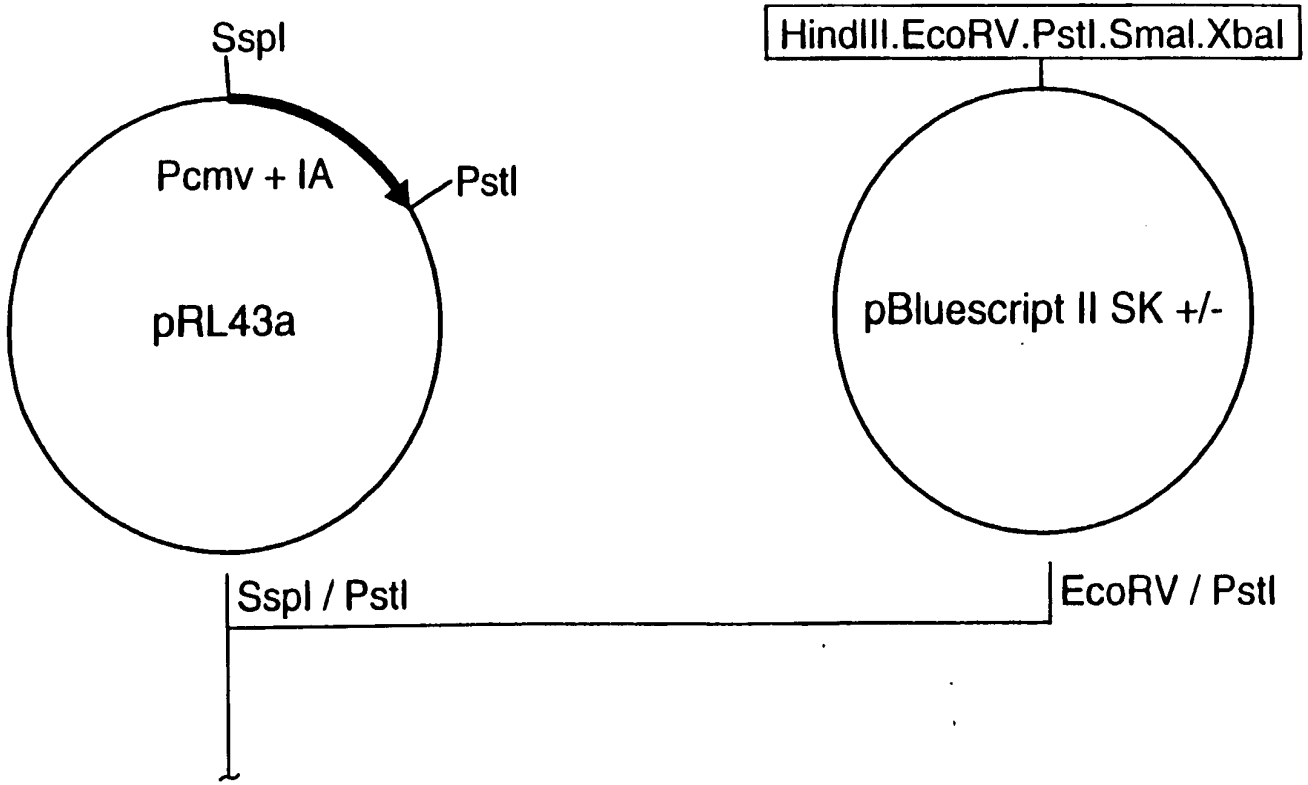

SspI
Pcmv + IA
PstI
pRL43a
SspI / PstI
HindIII.EcoRV.PstI.SmaI.XbaI
pBluescript II SK +/-
EcoRV / PstI

FIG.5A

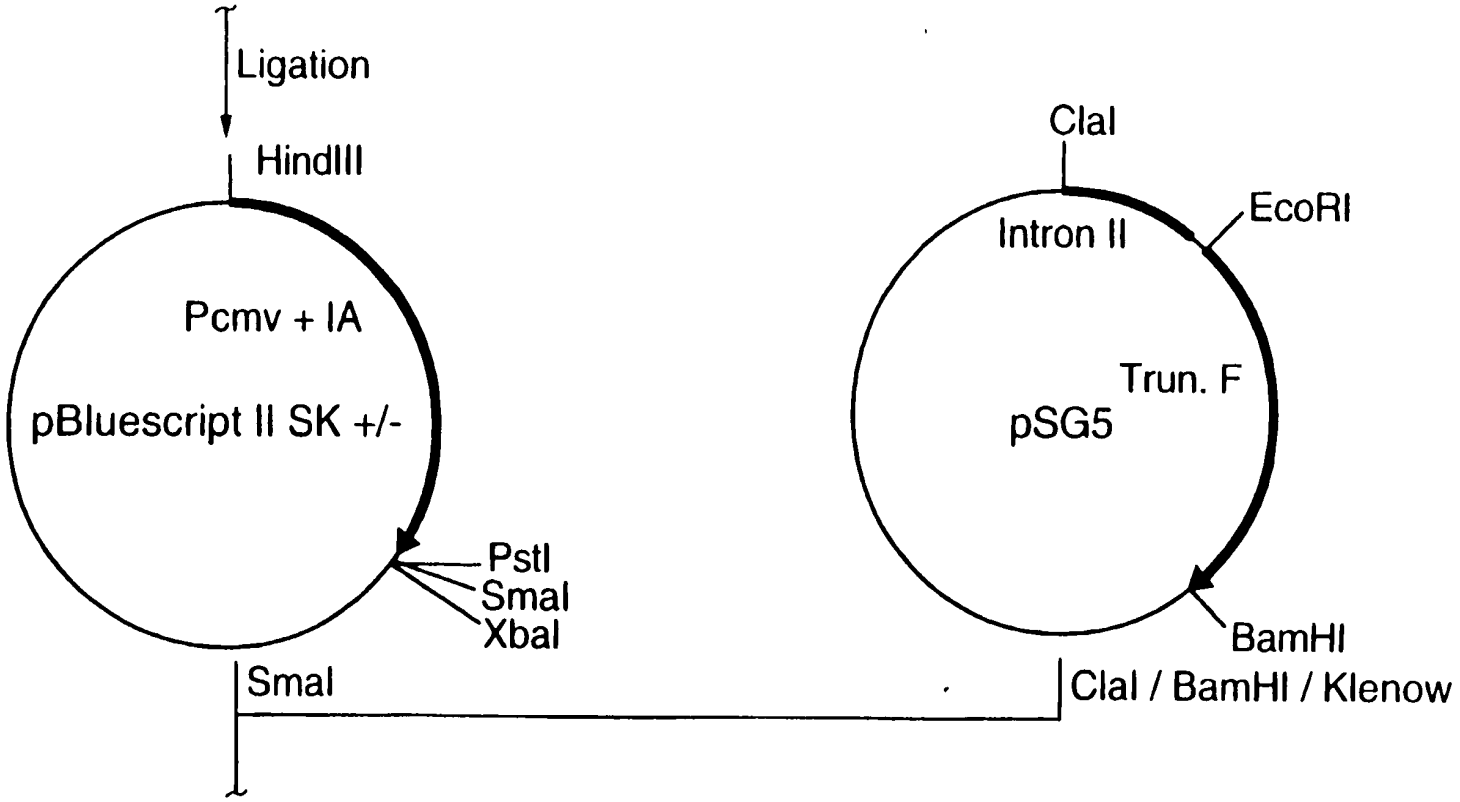

Ligation
HindIII
Pcmv + IA
pBluescript II SK +/-
PstI
SmaI
XbaI
SmaI
ClaI
EcoRI
Intron II
Trun. F
pSG5
BamHI
ClaI / BamHI / Klenow

FIG.5B

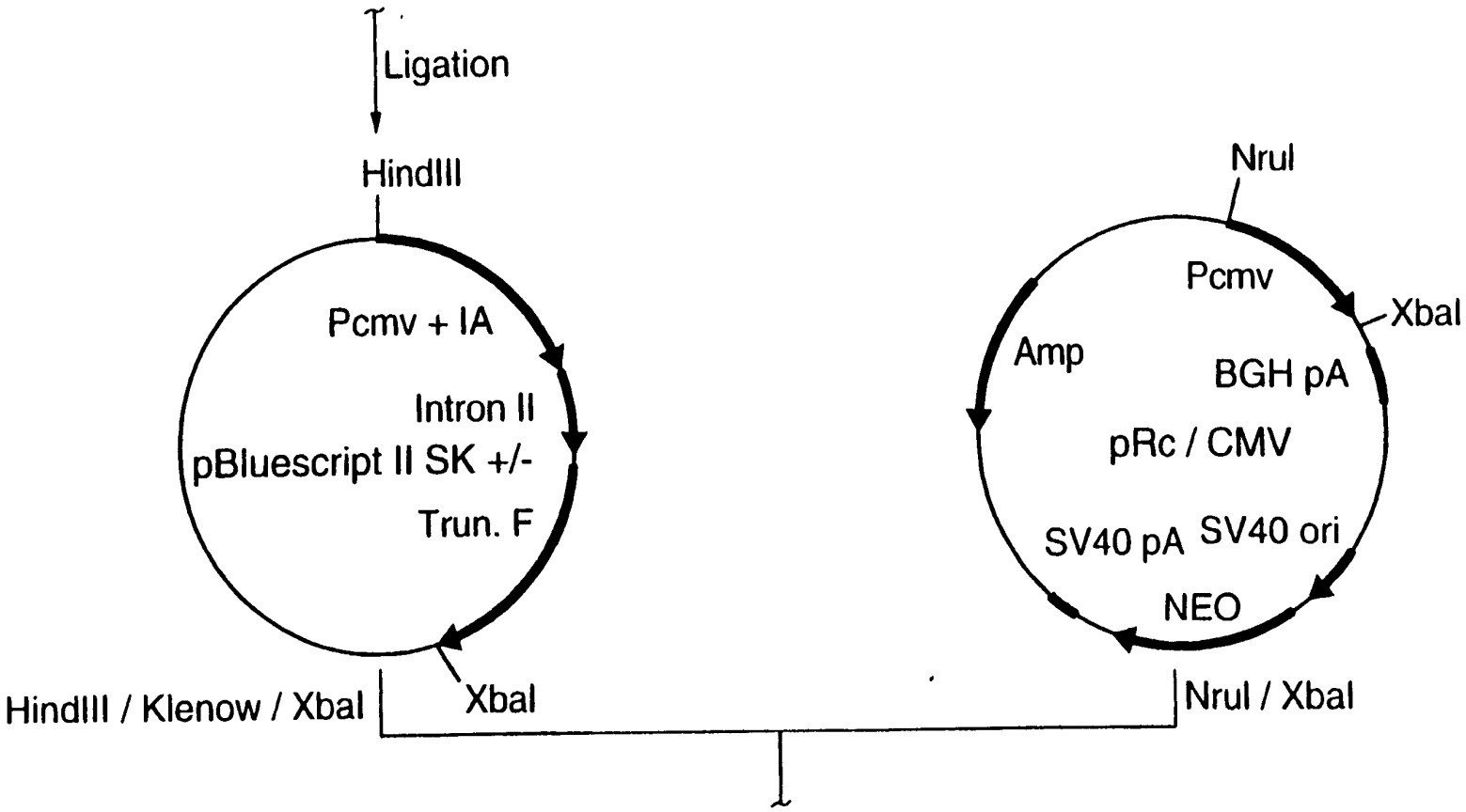

Ligation
HindIII
Pcmv + IA
Intron II
pBluescript II SK +/-
Trun. F
HindIII / Klenow / XbaI
XbaI
NruI
Pcmv
XbaI
Amp
BGH pA
pRc / CMV
SV40 pA
SV40 ori
NEO
NruI / XbaI

FIG.5C

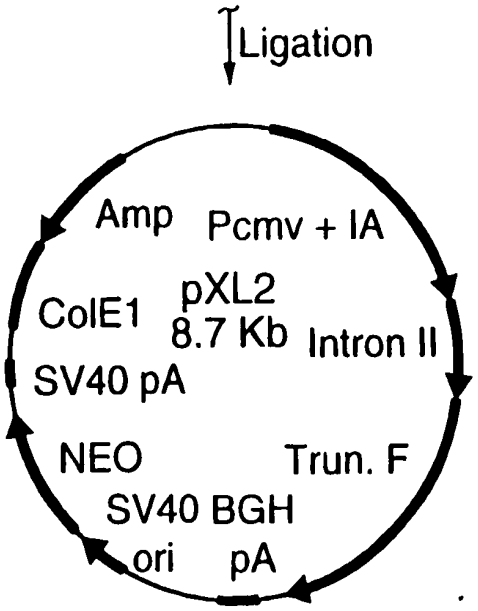

Ligation
Amp
Pcmv + IA
pXL2
8.7 Kb
ColE1
Intron II
SV40 pA
NEO
Trun. F
SV40 BGH
ori
pA

FIG.5D

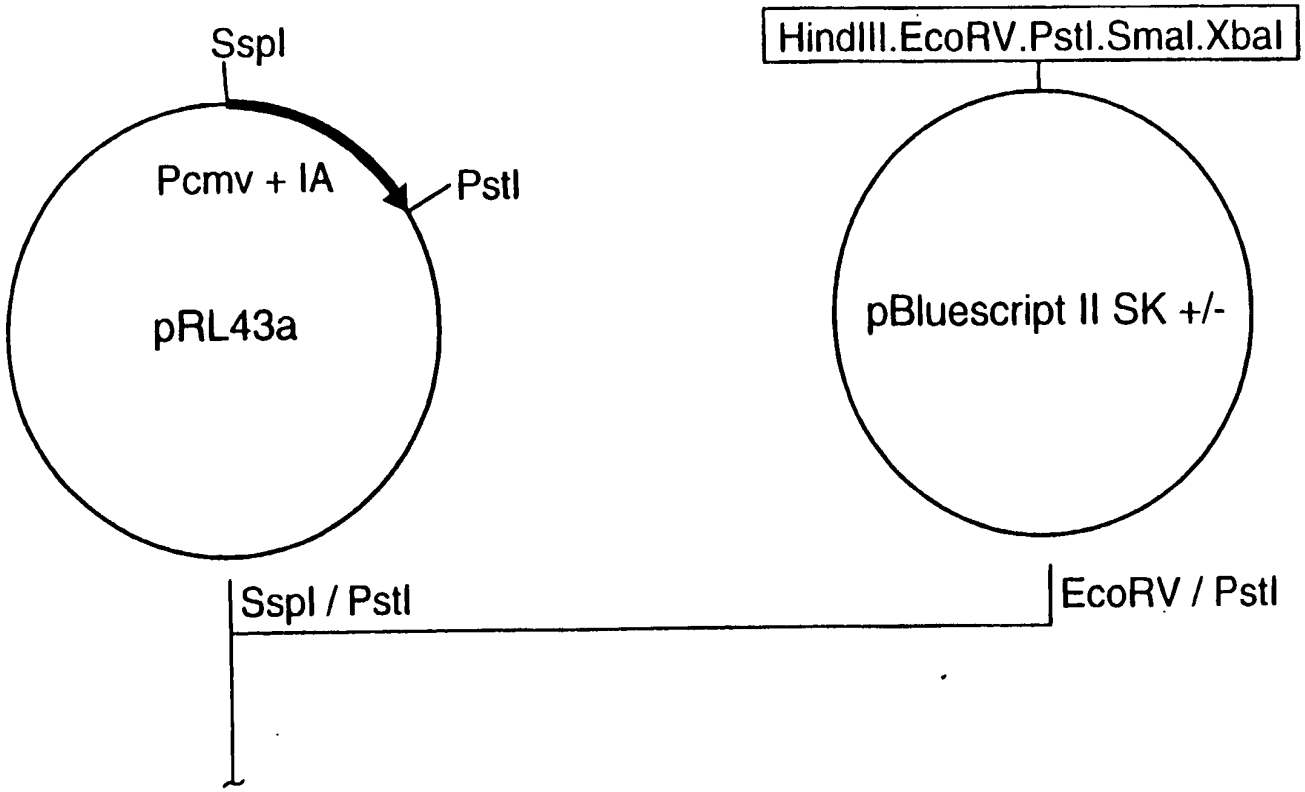

SspI
Pcmv + IA
PstI
pRL43a
HindIII.EcoRV.PstI.SmaI.XbaI
pBluescript II SK +/-
SspI / PstI
EcoRV / PstI

FIG.6A

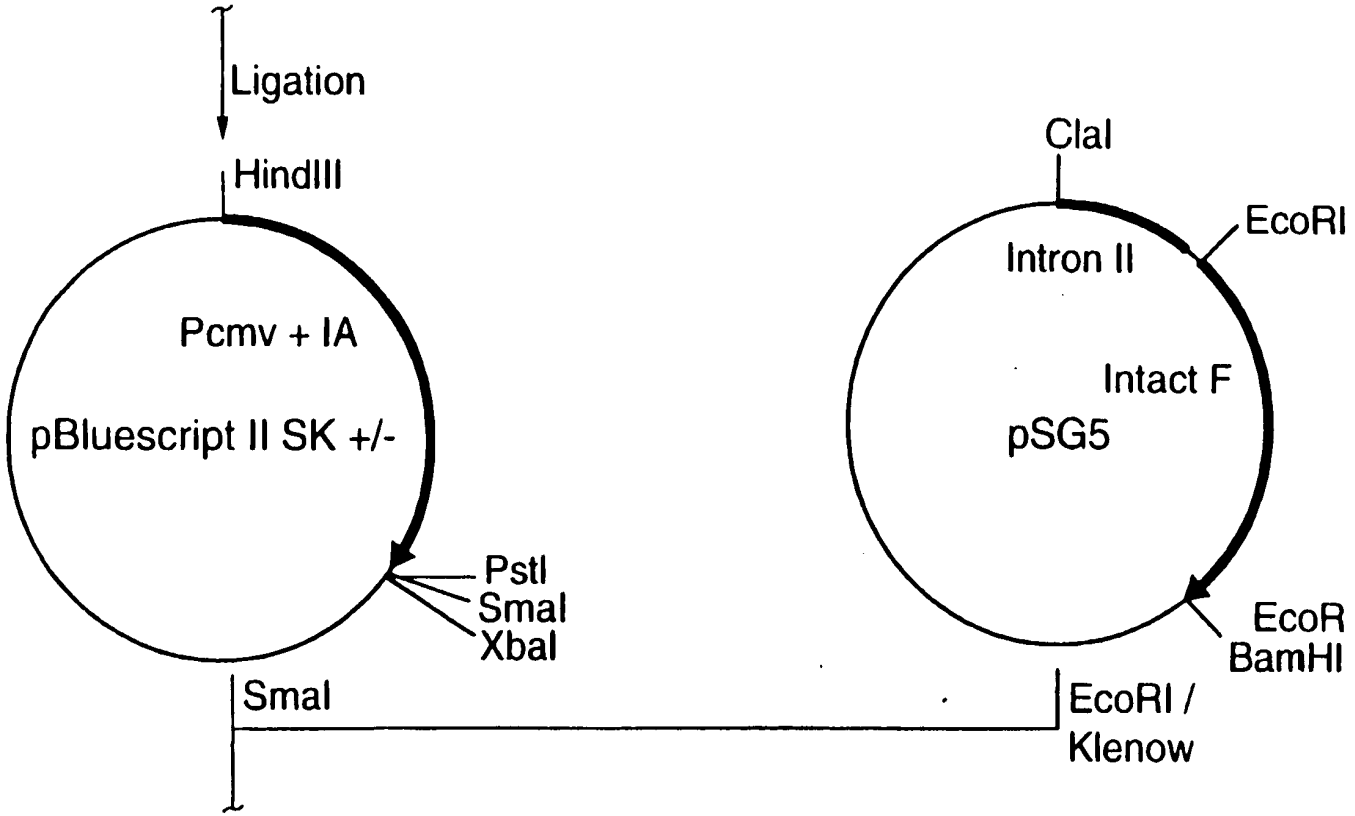

Ligation
HindIII
Pcmv + IA
pBluescript II SK +/-
PstI
SmaI
XbaI
SmaI
ClaI
EcoRI
Intron II
Intact F
pSG5
EcoR
BamHI
EcoRI /
Klenow

FIG.6B

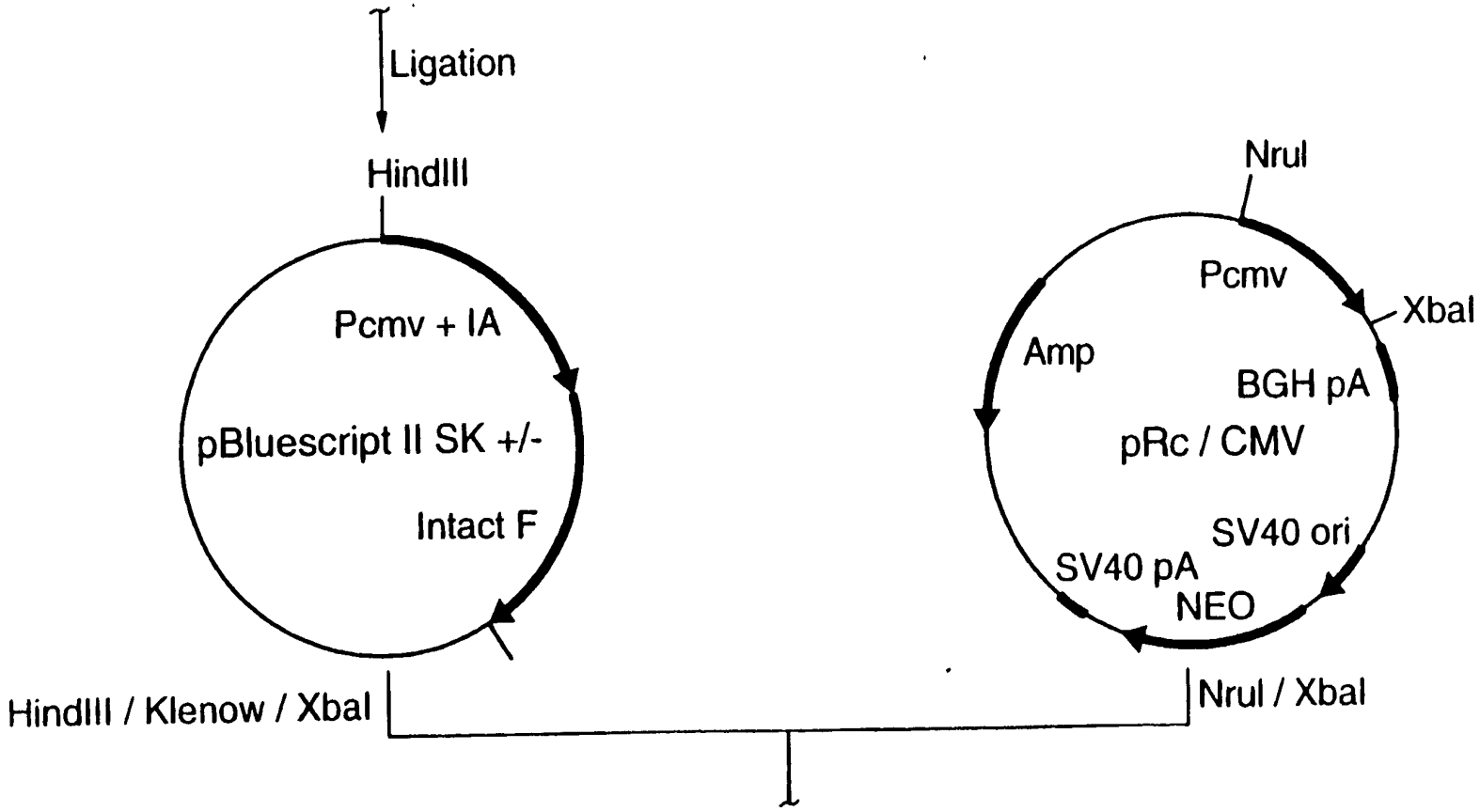

Ligation
HindIII
Pcmv + IA
pBluescript II SK +/-
Intact F
HindIII / Klenow / XbaI
NruI
Pcmv
XbaI
Amp
BGH pA
pRc / CMV
SV40 ori
SV40 pA
NEO
NruI / XbaI

FIG.6C

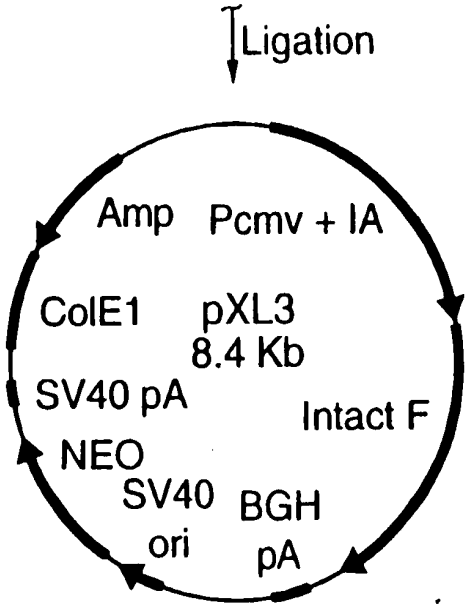

Ligation
Amp
Pcmv + IA
ColE1
pXL3
8.4 Kb
SV40 pA
Intact F
NEO
SV40
ori
BGH
pA

FIG.6D

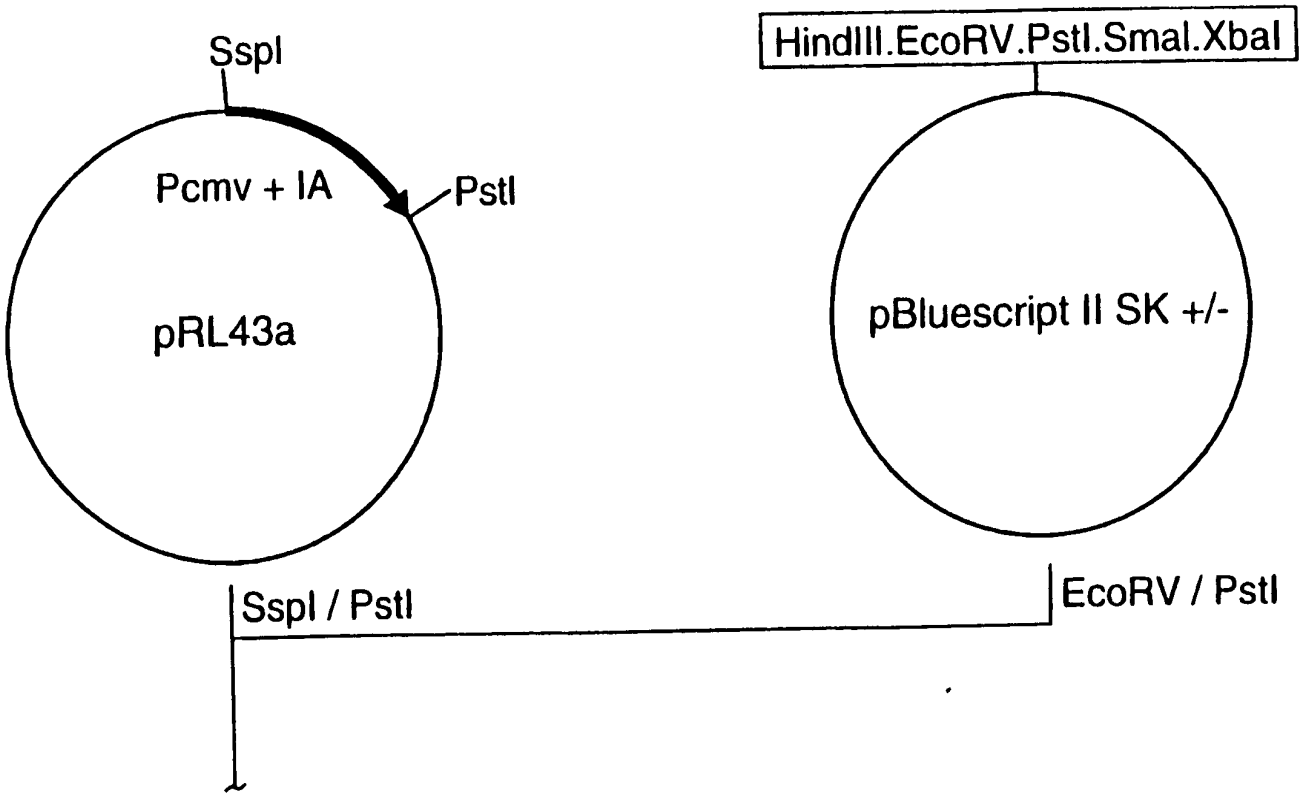

SspI
Pcmv + IA
PstI
pRL43a
HindIII.EcoRV.PstI.SmaI.XbaI
pBluescript II SK +/-
SspI / PstI
EcoRV / PstI

FIG.7A

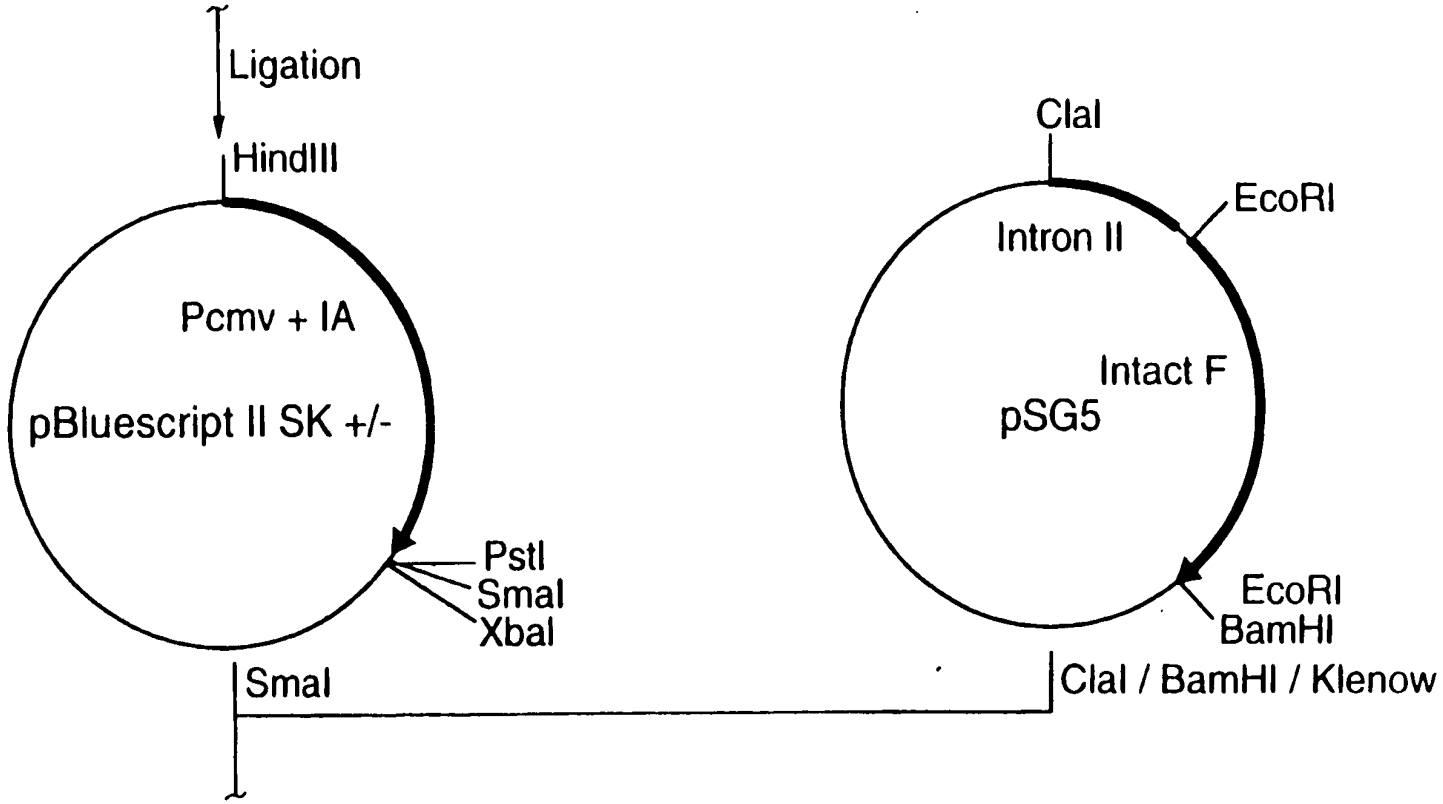

Ligation
HindIII
Pcmv + IA
pBluescript II SK +/-
PstI
SmaI
XbaI
SmaI
ClaI
EcoRI
Intron II
Intact F
pSG5
EcoRI
BamHI
ClaI / BamHI / Klenow

FIG.7B

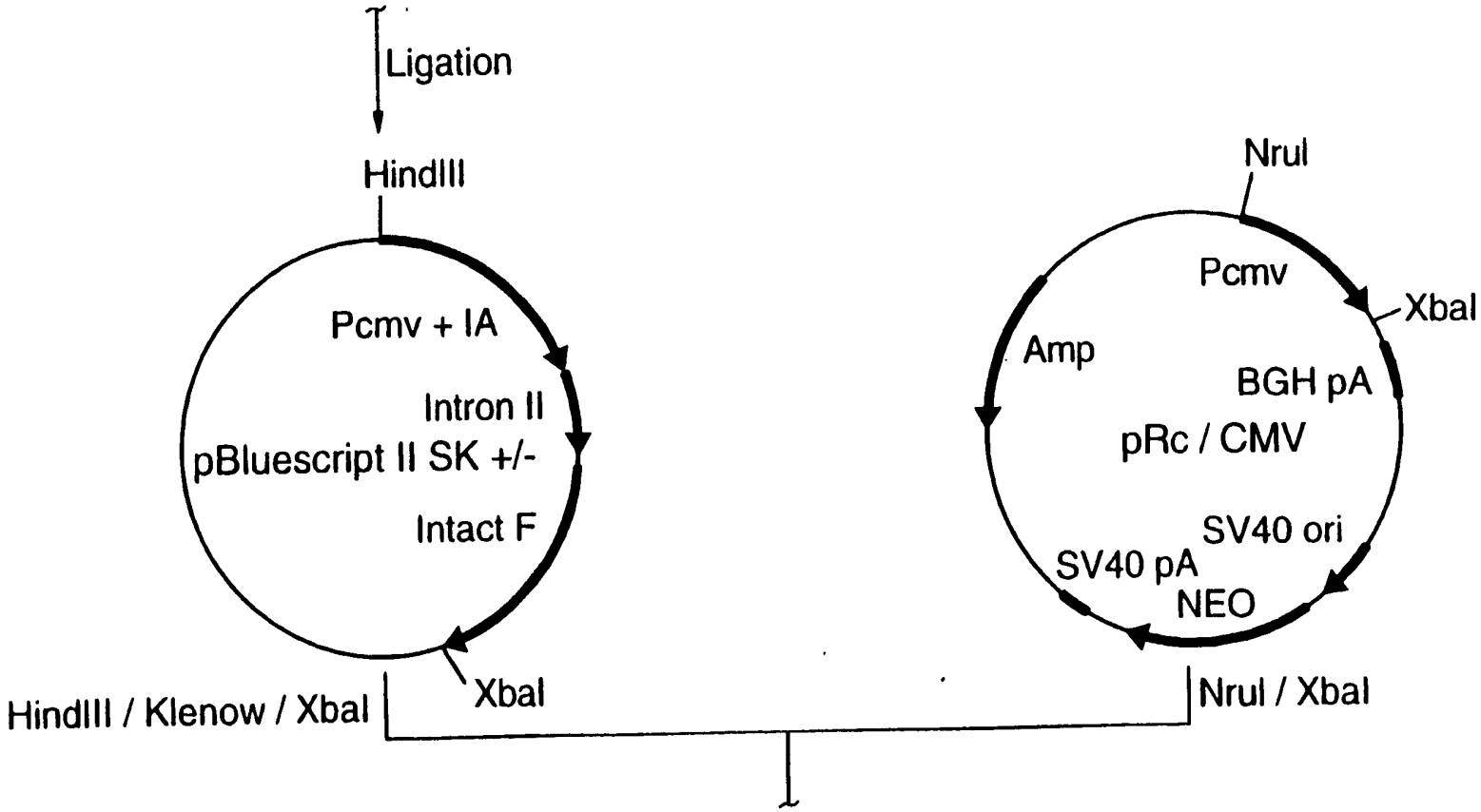

Ligation
HindIII
Pcmv + IA
Intron II
pBluescript II SK +/-
Intact F
XbaI
HindIII / Klenow / XbaI
NruI
Pcmv
XbaI
Amp
BGH pA
pRc / CMV
SV40 ori
SV40 pA
NEO
NruI / XbaI

FIG.7C

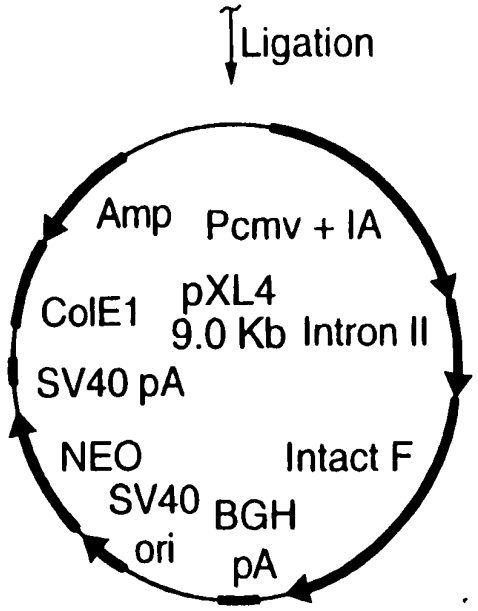

Ligation
Amp
Pcmv + IA
ColE1
pXL4
9.0 Kb
Intron II
SV40 pA
NEO
Intact F
SV40
ori
BGH
pA

FIG.7D

FIG.8

```
                                                                  GTGAGT
401 TTGGGGACCC TTGATTGTTC TTTCTTTTTC GCTATTGTAA AATTCATGTT
451 ATATGGAGGG GGCAAAGTTT TCAGGGTGTT GTTTAGAATG GGAAGATGTC
501 CCTTGTATCA CCATGGACCC TCATGATAAT TTTGTTTCTT TCACTTTCTA
551 CTCTGTTGAC AACCATTGTC TCCTCTTATT TTCTTTTCAT TTTCTGTAAC
601 TTTTTCGTTA AACTTTAGCT TGCATTTGTA ACGAATTTTT AAATTCACTT
651 TTGTTTATTT GTCAGATTGT AAGTACTTTC TCTAATCACT TTTTTTTCAA
701 GGCAATCAGG GTATATTATA TTGTACTTCA GCACAGTTTT AGAGAACAAT
751 TGTTATAATT AAATGATAAG GTAGAATATT TCTGCATATA AATTCTGGCT
801 GGCGTGGAAA TATTCTTATT GGTAGAAACA ACTACATCCT GGTCATCATC
851 CTGCCTTTCT CTTTATGGTT ACAATGATAT ACACTGTTTG AGATGAGGAT
901 AAAATACTCT GAGTCCAAAC CGGGCCCCTC TGCTAACCAT GTTCATGCCT
951 TCTTCTTTTT CCTACAG
```

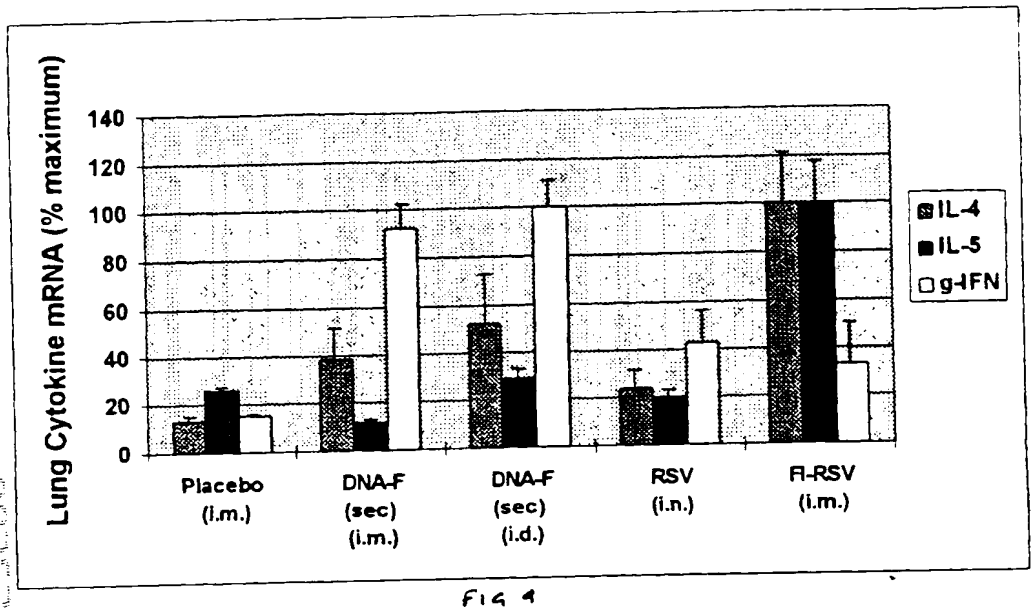

Lung Cytokine mRNA (% maximum)
140
120
100
80
60
40
20
0
Placebo (i.m.)
DNA-F (sec) (i.m.)
DNA-F (sec) (i.d.)
RSV (i.n.)
FI-RSV (i.m.)
IL-4
IL-5
g-IFN

Fig 4

Figure 10 Construction of p82M35B

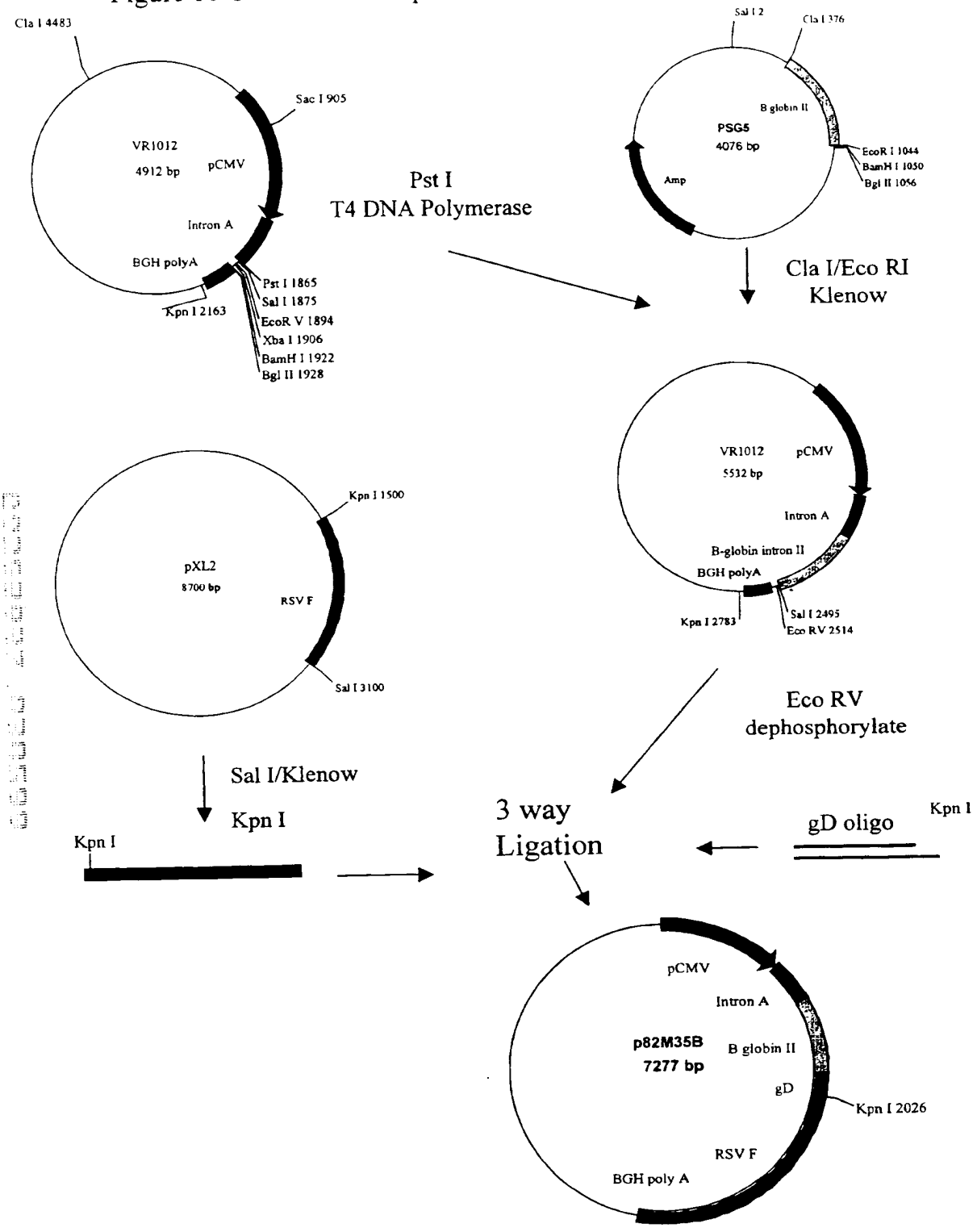


Figure II Nucleotide Sequence of plasmid VR1012

```
        10         20         30         40         50         60         70
TCGCGCGTTT CGGTGATGAC GGTGAAAACC TCTGACACAT GCAGCTCCCG GAGACGGTCA CAGCTTGTCT

        80         90        100        110        120        130        140
GTAAGCGGAT GCCGGGAGCA GACAAGCCCG TCAGGGCGCG TCAGCGGGTG TTGGCGGGTG TCGGGGCTGG

       150        160        170        180        190        200        210
CTTAACTATG CGGCATCAGA GCAGATTGTA CTGAGAGTGC ACCATATGCG GTGTGAAATA CCGCACAGAT

       220        230        240        250        260        270        280
GCGTAAGGAG AAAATACCGC ATCAGATTGG CTATTGGCCA TTGCATACGT TGTATCCATA TCATAATATG

       290        300        310        320        330        340        350
TACATTTATA TTGGCTCATG TCCAACATTA CCGCCATGTT GACATTGATT ATTGACTAGT TATTAATAGT

       360        370        380        390        400        410        420
AATCAATTAC GGGGTCATTA GTTCATAGCC CATATATGGA GTTCCGCGTT ACATAACTTA CGGTAAATGG

       430        440        450        460        470        480        490
CCCGCCTGGC TGACCGCCCA ACGACCCCCG CCCATTGACG TCAATAATGA CGTATGTTCC CATAGTAACG

       500        510        520        530        540        550        560
CCAATAGGGA CTTTCCATTG ACGTCAATGG GTGGAGTATT TACGGTAAAC TGCCCACTTG GCAGTACATC

       570        580        590        600        610        620        630
AAGTGTATCA TATGCCAAGT ACGCCCCCTA TTGACGTCAA TGACGGTAAA TGGCCCGCCT GGCATTATGC

       640        650        660        670        680        690        700
CCAGTACATG ACCTTATGGG ACTTTCCTAC TTGGCAGTAC ATCTACGTAT TAGTCATCGC TATTACCATG

       710        720        730        740        750        760        770
GTGATGCGGT TTTGGCAGTA CATCAATGGG CGTGGATAGC GGTTTGACTC ACGGGGATTT CCAAGTCTCC

       780        790        800        810        820        830        840
ACCCCATTGA CGTCAATGGG AGTTTGTTTT GGCACCAAAA TCAACGGGAC TTTCCAAAAT GTCGTAACAA

       850        860        870        880        890        900        910
CTCCGCCCCA TTGACGCAAA TGGGCGGTAG GCGTGTACGG TGGGAGGTCT ATATAAGCAG AGCTCGTTTA

       920        930        940        950        960        970        980
GTGAACCGTC AGATCGCCTG GAGACGCCAT CCACGCTGTT TTGACCTCCA TAGAAGACAC CGGGACCGAT

       990       1000       1010       1020       1030       1040       1050
CCAGCCTCCG CGGCCGGGAA CGGTGCATTG GAACGCGGAT TCCCCGTGCC AAGAGTGACG TAAGTACCGC

      1060       1070       1080       1090       1100       1110       1120
CTATAGACTC TATAGGCACA CCCCTTTGGC TCTTATGCAT GCTATACTGT TTTTGGCTTG GGGCCTATAC

      1130       1140       1150       1160       1170       1180       1190
ACCCCCGCTT CCTTATGCTA TAGGTGATGG TATAGCTTAG CCTATAGGTG TGGGTTATTG ACCATTATTG

      1200       1210       1220       1230       1240       1250       1260
ACCACTCCCC TATTGGTGAC GATACTTTCC ATTACTAATC CATAACATGG CTCTTTGCCA CAACTATCTC

      1270       1280       1290       1300       1310       1320       1330
TATTGGCTAT ATGCCAATAC TCTGTCCTTC AGAGACTGAC ACGGACTCTG TATTTTTACA GGATGGGGTC
```

```
      1340        1350        1360        1370        1380        1390        1400
CCATTTATTA TTTACAAATT CACATATACA ACAACGCCGT CCCCCGTGCC CGCAGTTTTT ATTAAACATA

      1410        1420        1430        1440        1450        1460        1470
GCGTGGGATC TCCACGCGAA TCTCGGGTAC GTGTTCCGGA CATGGGCTCT TCTCCGGTAG CGGCGGAGCT

      1480        1490        1500        1510        1520        1530        1540
TCCACATCCG AGCCCTGGTC CCATGCCTCC AGCGGCTCAT GGTCGCTCGG CAGCTCCTTG CTCCTAACAG

      1550        1560        1570        1580        1590        1600        1610
TGGAGGCCAG ACTTAGGCAC AGCACAATGC CCACCACCAC CAGTGTGCCG CACAAGGCCG TGGCGGTAGG

      1620        1630        1640        1650        1660        1670        1680
GTATGTGTCT GAAAATGAGC GTGGAGATTG GGCTCGCACG GCTGACGCAG ATGGAAGACT TAAGGCAGCG

      1690        1700        1710        1720        1730        1740        1750
GCAGAAGAAG ATGCAGGCAG CTGAGTTGTT GTATTCTGAT AAGAGTCAGA GGTAACTCCC GTTGCGGTGC

      1760        1770        1780        1790        1800        1810        1820
TGTTAACGGT GGAGGGCAGT GTAGTCTGAG CAGTACTCGT TGCTGCCGCG CGCGCCACCA GACATAATAG

      1830        1840        1850        1860        1870        1880        1890
CTGACAGACT AACAGACTGT TCCTTTCCAT GGGTCTTTTC TGCAGTCACC GTCGTCGACA CGTGTGATCA

      1900        1910        1920        1930        1940        1950        1960
GATATCGCGG CCGCTCTAGA CCAGGCGCCT GGATCCAGAT CTGCTGTGCC TTCTAGTTGC CAGCCATCTG

      1970        1980        1990        2000        2010        2020        2030
TTGTTTGCCC CTCCCCCGTG CCTTCCTTGA CCCTGGAAGG TGCCACTCCC ACTGTCCTTT CCTAATAAAA

      2040        2050        2060        2070        2080        2090        2100
TGAGGAAATT GCATCGCATT GTCTGAGTAG GTGTCATTCT ATTCTGGGGG GTGGGGTGGG GCAGGACAGC

      2110        2120        2130        2140        2150        2160        2170
AAGGGGGAGG ATTGGGAAGA CAATAGCAGG CATGCTGGGG ATGCGGTGGG CTCTATGGGT ACCCAGGTGC

      2180        2190        2200        2210        2220        2230        2240
TGAAGAATTG ACCCGGTTCC TCCTGGGCCA GAAAGAAGCA GGCACATCCC CTTCTCTGTG ACACACCCTG

      2250        2260        2270        2280        2290        2300        2310
TCCACGCCCC TGGTTCTTAG TTCCAGCCCC ACTCATAGGA CACTCATAGC TCAGGAGGGC TCCGCCTTCA

      2320        2330        2340        2350        2360        2370        2380
ATCCCACCCG CTAAAGTACT TGGAGCGGTC TCTCCCTCCC TCATCAGCCC ACCAAACCAA ACCTAGCCTC

      2390        2400        2410        2420        2430        2440        2450
CAAGAGTGGG AAGAAATTAA AGCAAGATAG GCTATTAAGT GCAGAGGGAG AGAAAATGCC TCCAACATGT

      2460        2470        2480        2490        2500        2510        2520
GAGGAAGTAA TGAGAGAAAT CATAGAATTT CTTCCGCTTC CTCGCTCACT GACTCGCTGC GCTCGGTCGT

      2530        2540        2550        2560        2570        2580        2590
TCGGCTGCGG CGAGCGGTAT CAGCTCACTC AAAGGCGGTA ATACGGTTAT CCACAGAATC AGGGGATAAC

      2600        2610        2620        2630        2640        2650        2660
GCAGGAAAGA ACATGTGAGC AAAAGGCCAG CAAAAGGCCA GGAACCGTAA AAAGGCCGCG TTGCTGGCGT

      2670        2680        2690        2700        2710        2720        2730
TTTTCCATAG GCTCCGCCCC CCTGACGAGC ATCACAAAAA TCGACGCTCA AGTCAGAGGT GGCGAAACCC
```

```
      2740        2750        2760        2770        2780        2790        2800
GACAGGACTA TAAAGATACC AGGCGTTTCC CCCTGGAAGC TCCCTCGTGC GCTCTCCTGT TCCGACCCTG

      2810        2820        2830        2840        2850        2860        2870
CCGCTTACCG GATACCTGTC CGCCTTTCTC CCTTCGGGAA GCGTGGCGCT TTCTCATAGC TCACGCTGTA

      2880        2890        2900        2910        2920        2930        2940
GGTATCTCAG TTCGGTGTAG GTCGTTCGCT CCAAGCTGGG CTGTGTGCAC GAACCCCCCG TTCAGCCCGA

      2950        2960        2970        2980        2990        3000        3010
CCGCTGCGCC TTATCCGGTA ACTATCGTCT TGAGTCCAAC CCGGTAAGAC ACGACTTATC GCCACTGGCA

      3020        3030        3040        3050        3060        3070        3080
GCAGCCACTG GTAACAGGAT TAGCAGAGCG AGGTATGTAG GCGGTGCTAC AGAGTTCTTG AAGTGGTGGC

      3090        3100        3110        3120        3130        3140        3150
CTAACTACGG CTACACTAGA AGAACAGTAT TTGGTATCTG CGCTCTGCTG AAGCCAGTTA CCTTCGGAAA

      3160        3170        3180        3190        3200        3210        3220
AAGAGTTGGT AGCTCTTGAT CCGGCAAACA AACCACCGCT GGTAGCGGTG GTTTTTTTGT TTGCAAGCAG

      3230        3240        3250        3260        3270        3280        3290
CAGATTACGC GCAGAAAAAA AGGATCTCAA GAAGATCCTT TGATCTTTTC TACGGGGTCT GACGCTCAGT

      3300        3310        3320        3330        3340        3350        3360
GGAACGAAAA CTCACGTTAA GGGATTTTGG TCATGAGATT ATCAAAAAGG ATCTTCACCT AGATCCTTTT

      3370        3380        3390        3400        3410        3420        3430
AAATTAAAAA TGAAGTTTTA AATCAATCTA AAGTATATAT GAGTAAACTT GGTCTGACAG TTACCAATGC

      3440        3450        3460        3470        3480        3490        3500
TTAATCAGTG AGGCACCTAT CTCAGCGATC TGTCTATTTC GTTCATCCAT AGTTGCCTGA CTCGGGGGGG

      3510        3520        3530        3540        3550        3560        3570
GGGGGCGCTG AGGTCTGCCT CGTGAAGAAG GTGTTGCTGA CTCATACCAG GCCTGAATCG CCCCATCATC

      3580        3590        3600        3610        3620        3630        3640
CAGCCAGAAA GTGAGGGAGC CACGGTTGAT GAGAGCTTTG TTGTAGGTGG ACCAGTTGGT GATTTTGAAC

      3650        3660        3670        3680        3690        3700        3710
TTTTGCTTTG CCACGGAACG GTCTGCGTTG TCGGGAAGAT GCGTGATCTG ATCCTTCAAC TCAGCAAAAG

      3720        3730        3740        3750        3760        3770        3780
TTCGATTTAT TCAACAAAGC CGCCGTCCCG TCAAGTCAGC GTAATGCTCT GCCAGTGTTA CAACCAATTA

      3790        3800        3810        3820        3830        3840        3850
ACCAATTGTG ATTAGAAAAA CTCATCGAGC ATCAAATGAA ACTGCAATTT ATTCATATCA GGATTATCAA

      3860        3870        3980        3890        3900        3910        3920
TACCATATTT TTGAAAAAGC CGTTTCTGTA ATGAAGGAGA AAACTCACCG AGGCAGTTCC ATAGGATGGC

      3930        3940        3950        3960        3970        3980        3990
AAGATCCTGG TATCGGTCTG CGATTCCGAC TCGTCCAACA TCAATACAAC CTATTAATTT CCCCTCGTCA

      4000        4010        4020        4030        4040        4050        4060
AAAATAAGGT TATCAAGTGA GAAATCACCA TGAGTGACGA CTGAATCCGG TGAGAATGGC AAAAGCTTAT

      4070        4080        4090        4100        4110        4120        4130
GCATTTCTTT CCAGACTTGT TCAACAGGCC AGCCATTACG CTCGTCATCA AAATCACTCG CATCAACCAA
```

```
      4140        4150        4160        4170        4180        4190        4200
ACCGTTATTC ATTCGTGATT GCGCCTGAGC GAGACGAAAT ACGCGATCGC TGTTAAAAGG ACAATTACAA

      4210        4220        4230        4240        4250        4260        4270
ACAGGAATCG AATGCAACCG GCGCAGGAAC ACTGCCAGCG CATCAACAAT ATTTTCACCT GAATCAGGAT

      4280        4290        4300        4310        4320        4330        4340
ATTCTTCTAA TACCTGGAAT GCTGTTTTCC CGGGGATCGC AGTGGTGAGT AACCATGCAT CATCAGGAGT

      4350        4360        4370        4380        4390        4400        4410
ACGGATAAAA TGCTTGATGG TCGGAAGAGG CATAAATTCC GTCAGCCAGT TTAGTCTGAC CATCTCATCT

      4420        4430        4440        4450        4460        4470        4480
GTAACATCAT TGGCAACGCT ACCTTTGCCA TGTTTCAGAA ACAACTCTGG CGCATCGGGC TTCCCATACA

      4490        4500        4510        4520        4530        4540        4550
ATCGATAGAT TGTCGCACCT GATTGCCCGA CATTATCGCG AGCCCATTTA TACCCATATA AATCAGCATC

      4560        4570        4580        4590        4600        4610        4620
CATGTTGGAA TTTAATCGCG GCCTCGAGCA AGACGTTTCC CGTTGAATAT GGCTCATAAC GTTCCTTGTA

      4630        4640        4650        4660        4670        4680        4690
TTACTGTTTA TGTAAGCAGA CAGTTTTATT GTTCATGATG ATATATTTTT ATCTTGTGCA ATGTAACATC

      4700        4710        4720        4730        4740        4750        4760
AGAGATTTTG AGACACAACG TGGCTTTCCC CCCCCCCCCA TTATTGAAGC ATTTATCAGG GTTATTGTCT

      4770        4780        4790        4800        4810        4820        4830
CATGAGCGGA TACATATTTG AATGTATTTA GAAAAATAAA CAAATAGGGG TTCCGCGCAC ATTTCCCCGA

      4840        4850        4860        4870        4880        4890        4900
AAAGTGCCAC CTGACGTCTA AGAAACCATT ATTATCATGA CATTAACCTA TAAAAATAGG CGTATCACGA

      4910
GGCCCTTTCG TC
```

FIGURE 12

5'AAG CTT CAG GAA CGA CCA ACT ACC CCG ATC ATC AGT TAT CCT

TAA GGT CTC TTT TGT GTG GTG CGT TCC GGT ATG GGG GGG ACT GCC

Met Gly Gly Thr Ala

GCC AGG TTG GGG GCC GTG ATT TTG TTT GTC GTC ATA GTG GGC CTC

Ala Arg Leu Gly Ala Val Ile Leu Phe Val Val Ile Val Gly Leu

CAT GGG GTC CGC GGC AAA TAT GCC TTG GCG GAT GCC TCT CTC 3'

His Gly Val Arg Gly Lys Tyr Ala Leu Ala Asp Ala Ser Leu